

EXPRESSION AND SECRETION OF FOREIGN PROTEINS BY 'ASPERGILLUS'

Norma Lucena Cavalcanti Licinio Da Silva

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**Expression and Secretion
of foreign proteins
by *Aspergillus***

Norma Lucena Cavalcanti Licinio da Silva

Submitted in accordance with the requirements for the degree
of Doctor of Philosophy

University of St. Andrews
School of Biological and Medical Sciences

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DECLARATION

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SUMMARY

The feasibility of using the ascomycetes filamentous fungus *Aspergillus oryzae* as a host organism for foreign protein expression and secretion was assessed. For this purpose, human interleukin-6 (hIL-6) and the Simian virus 5 haemagglutinin-neuraminidase (HN) were used as model foreign proteins.

Expression and secretion of the human interleukin 6 gene have been achieved using vector construction containing the entire *A.niger glaA* coding region linked to the hIL-6 cDNA through a KEX2-motif encoding DNA. hIL-6 containing expression vectors were introduced into *A.oryzae* nitrate non-utilising mutant strains *niaD14*, *niaD200*, *niaD500* (*niaD*⁻) and arginine auxotrophic strain 1560-6 (*argB*⁻) by means of genetic cotransformation technology. The *A.oryzae niaD* gene or the *A.nidulans argB* or *amdS* genes were used as the transfer and selection system. *A.oryzae* nitrate and acetamide prototrophic transformants, containing the hIL-6 DNA sequence, failed to secrete free hIL-6 molecules. Recombinant hIL-6 protein was detected in culture filtrates from *A.oryzae* 1560-6 arginine prototrophic transformants T1560-gla15 and T1560-gpd1. The results suggested that *A.oryzae* proteases recognized the KEX2-

motif yielding free hIL-6 molecules. However, hIL-6 was found to be selective degraded by *A.oryzae* proteases compared with the heterologous glucoamylase (GLAA). The incubation time for hIL-6 detection has been shown to vary with the host strain and growth conditions. Hybrid GLAA/hIL-6 protein was also found to be secreted. Improvement in hIL-6 yields were obtained by generating 2-deoxy-D-glucose resistant mutants of transformant T1560-gla15 and by manipulating the host growth conditions including the use of microbial solid state culture. The maximum level of secreted hIL-6 was found to be approximately 4 mg/l as judged from SDS-PAGE polyacrylamide gels and western blots analysis.

The Simian virus 5 Haemagglutinin-neuraminidase (HN) glycosylation, folding and oligomerization characteristics have been well studied in mammalian cells. Therefore, HN is an ideal model protein, *a priori*, for studying secretion / processing in foreign hosts including filamentous fungi. For such studies the entire HN cDNA sequence (designated HN-DNA fragment) was generated by PCR technology. However, as the HN protein is a membrane bound glycoprotein type II, HN might not be secreted due to its anchor-domain, thus a deletion of the first 108 bp of its cDNA sequence, corresponding to the hydrophobic N-terminal region, was carried out in a second PCR reaction (designated ASSHN-DNA fragment).

Both DNA fragments, encoding HN or ASSHN protein sequences, were inserted in-frame downstream of the *A.niger glaA* coding region in plasmid vector pAN56-30, generating pAN56-HN and pAN56-ASSHN. Additionally, both DNA fragments were inserted in-frame downstream of the *A.oryzae amy3* gene present in vector pSTA1210, generating pSTA1210-HN and pSTA1210-ASSHN. These expression cassettes were introduced into *A.oryzae* 1560-6, an amylase over-producing industrial strain, by means of cotransformation. The *A.nidulans argB* and *amdS* genes were used as transfer and selection systems. The HN protein was found in culture filtrates of arginine prototrophic transformants harbouring the *glaA*/ASSHN and *amy3*/ASSHN DNA sequences. HN protein was mainly detected by immunoprecipitation of 50 ml of culture filtrate. Such low expression level do not seem to be related to gene copy number, but rather to proteolytic degradation. Another possibility is that HN was not translated efficiently. Improvement in HN yield was achieved by cultivation on solid state culture in which HN protein was detected in 1 ml aliquot of culture filtrate as determine by western blotting. Nevertheless, the yield of HN protein extracted with 50 ml of sterile distilled water from 10 g rice solid state culture is estimated to be less than 1 μ g per ml as judged by the western blots.

ABBREVIATIONS

α	alpha
A	adenine
A _{260nm}	absorbance at 260 nm
A _{280nm}	absorbance at 280 nm
aa	amino acids
ADP	adenosine diphosphate
<i>a priori</i>	first concern
ASSHN	Δ SSHN
Δ SSHN	HN protein without leader sequence
ATP	adenosine triphosphate
β	beta
BCIP	5-bromo-4-chloro-3-indoly phosphate
bp	base pair(s)
BSA	bovine serum albumin
C	cytosine
C-	carbon
°C	degrees centigrade
cm	centimetres
CM	complete medium
Da	Dalton
dATP	2'-deoxy-adenosine-5'-triphosphate
dC7GTP	methyl dGTP
dCTP	2'-deoxy-cytosine-5'-triphosphate
ddNTPs	2',3'-dideoxynucleoside 5'triphosphates
DEPC	diethylpyrocarbonate
dGTP	2'-deoxy-guanosine-5'-triphosphate
dH ₂ O	distilled water
dITP	2'-deoxy-inosine-5'-triphosphate
DMSO	dimethyl sulfoxide
DNA	deoxyribose nucleic acid
DNase	deoxyribonuclease
dNTP(s)	deoxynucleotides

dsDNA	double stranded DNA
DTT	dithiothreitol
dTTP	2'-deoxy-thimine-5'-triphosphate
EDTA	ethylene diamine tetra-acetic acid
<i>e.g.</i>	<i>exempli gratia</i> (for example)
ER	endoplasmic reticulum
<i>et al.</i>	<i>et alia</i> (and others)
EtBr	ethidium bromide
g	gram(s)
G	guanine
GLAA	<i>A.niger</i> glucoamylase protein
h	hour(s)
HN	haemagglutinin-neuraminidase protein
hIL6	human interleukin 6
human IL-6	human interleukin 6
<i>i.e.</i>	<i>id est</i> (that is)
IgG	immunoglobulin G
IMI	fungal strains from British collection
Inc.	Incorporation
IPTG	isopropyl β -thiogalactoside
x g	times force of gravity
k	1 thousand
kb	kilobase pairs
kDa	kiloDalton
l	litre
LB	Luria broth
M	molar
mA	milliampere
MAb(s)	monoclonal antibody
μ Ci	microCuries
μ g	micrograms
mg	milligrams
min	minutes
MOPS	morpholinopropanesulfonic acid
μ l	microlitres
ml	millilitres

mm	millimetres
mM	millimolar
MM	minimal media
mRNA	messenger RNA
M	molecular weight
N-	nitrogen
NBT	nitro blue tetrazolium
nm	nanometres
OD	optical density (wavelength)
pAb(s)	polyclonal antibody
PABA	para amino benzoic acid
PAGE	polyacrylamide gel electrophoresis
PBS	10 mM phosphate buffer pH7.4; 0.9% NaCl
PCR	polymerase chain reaction
PEG	polyethylene glycol
pg	picograms
pMol	picomoles
PMSF	phenylmethanesulphonyl fluoride
RIB	fungal strains from Japanese collection
RNA	ribose nucleic acid
RNase	ribonuclease
RPM	revolutions per minute
sec	second(s)
ssDNA	single strand DNA
SDS	sodium dodecyl sulphate
SDW	sterile distilled water
SS	signal sequence
SSC	3 M NaCl, 0.3 M trisodium citrate
SSPE	3.6 M NaCl, 0.2M sodium phosphate, 0.02 M EDTA, pH 7.7
T	thymine
TAE	0.04 M Tris-acetate, 0.002 M EDTA
TBE	0.089 M Tris-Borate, 0.089 M boric acid
TE	10 mM Tris-HCl pH 8.0, 1 mM EDTA
TEMED	N,N,N',N'-tetramethylethylene-diamine
TmS	melting temperature strand

Topt	optimal temperature
Tris	Tris (hydroxymethyl) aminomethane
TRITON X100	octyl phenoxy polyethoxyethanol
Tss	strand separation temperature
TWEEN 20	polyoxyethylene sobitan monolaurate
TWEEN 80	polyoxyethylene sobitan monooleate
U	units
UV	ultraviolet light
V	volts
v/v	volume per volume
w/v	weight per volume
WT	wild type
x	times
X	any nucleotide
x g	times force of gravity
X-gal	5'-bromo-4'-chloro-3'-indolyl- β -D-galactopyranoside
yeast	<i>Saccharomyces cerevisiae</i>
>	greater than
<	smaller than
&	and

Amino acids abbreviation:

<u>Amino acid</u>	<u>Three letter code</u>	<u>One letter code</u>
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N, B
Aspartic acid	Asp	D, B
Cysteine	Cys	C
Glutamic acid	Glu	E, Z
Glutamine	Gln	Q, Z
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V

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CHAPTER 1

INTRODUCTION

I GENERAL INTRODUCTION

For many centuries, the metabolic capacity of microorganisms has been used in the traditional manufacture of food and beverages (reviewed by Wood & Min, 1975). In the present century, a wide range of microbial compounds of industrial interest such as enzymes, organic acids and antibiotics has been recognized (reviewed by Miall, 1975; Adler-Nissen, 1987; Berka et al., 1991). However, commercial production of these compounds were often limited, as microorganisms, in general, use their biochemical machinery to synthesise metabolites and cell constituents efficiently, i.e. in the minimal amounts required for maintenance and growth. In the 1950s, the demand for microbial products, such as antibiotics and enzymes, increased. Improvements in industrial production processes were the first approach towards to increased production of microbial compounds (reviewed by Lockwood, 1975; Blain, 1975).

In the early 1970s, the advent of recombinant DNA technology took place. Methods for the isolation,

characterization, *in vitro* manipulation, transfer and reintegration of genetic material was first developed in the bacterium *Escherichia coli* (for reviews see Carbon *et al.*, 1977; Bolivar *et al.*, 1977; Watson *et al.*, 1983). A few years later transformation systems and the construction of DNA cloning vectors were reported in *Saccharomyces cerevisiae* (Beggs, 1978; Morris *et al.*, 1981) and filamentous fungi (Case *et al.*, 1979; Tilburn *et al.*, 1983; Ballance *et al.*, 1983; Yelton *et al.*, 1984). Consequently, the expression of specific microbial genes in an induced and controlled manner has permitted commercial production of microbial proteins (Sakaguchi *et al.*, 1992; Martin & Gutierrez, 1992). Additionally, the increased knowledge of gene regulation has provided the basis for investigating the possibility of further improvements in the current production processes of important microbial products (Felenbok, 1991; Renno *et al.*, 1992; Verdoes *et al.*, 1993). The one important feature of genetic manipulation is the possibility of altering the microbial biochemical machinery to allow the production of heterologous proteins, especially mammalian proteins of pharmaceutical interest, such as somatostatin (Green *et al.*, 1986b), human epidermal growth factor (Brake *et al.*, 1984), insulin (Thim *et al.*, 1986, 1989), and interleukin-6 (Contreras *et al.*, 1991). There are two major reasons for expressing a protein in a heterologous

host. The first is commercial. The availability of certain proteins, such as calf chymosin, may be erratic and so high levels of expression in a microbial host can fulfill demand. Moreover, the heterologous expression of therapeutically useful proteins such as interferon $\alpha 2$ (Hitzeman et al., 1983 and MacRae et al., 1993) and tissue plasminogen activator (Gwynne & Devchand, 1991) can be profitable even if they are produced at low levels. The second reason is academic. Heterologous expression can provide data of structure and/or function of a protein either in terms of biological activity or protein engineering as well as secretion processes *per se*. However, efficient heterologous gene expression requires an appropriate expression system which consists of the three major components: (1) an appropriate host system, (2) a transfer system and (3) an expression cassette .

1.1 Host system available

The bacterium *Escherichia coli* (Itakura et al., 1977; Villa-Komaroff et al., 1978) and the yeast *Saccharomyces cerevisiae* (Hitzeman et al., 1981; Bitter & Egan, 1984) were the first microorganisms assessed for the production of recombinant proteins, as a consequence

of the extensive knowledge of their genetics and in particular their regulation of gene expression .

There are several advantages to using bacteria, such as *E.coli*, for the expression of heterologous proteins. First, a large number of expression vectors are available. Second, their high specific growth rate might lead to high levels of eukaryotic gene transcription and translation, resulting in the production of large amounts of foreign proteins. Third, the potential loss of the expression vector in large scale fermentation can be avoided by growing the recombinant cells in selective media in which only cells carrying the selective markers encoded in plasmid DNA survive. However, bacteria do not appear to perform post-translational modifications such as glycosylation and disulfide bridge formation, which might affect the solubility, activity and stability of the produced protein and render it more susceptible to proteolytic degradation (Goodson & Katre, 1990; Bitter, 1986). Furthermore, the processing of recombinant eukaryotic protein might be carried out differently in prokaryotic cells, resulting in a product with reduced or no biological activity and / or with potential immunogenicity problems (Ben-Bassat & Bauer, 1987). In addition, proteins produced in Gram negative bacteria accumulate inside the cytoplasm forming with homologous

proteins and fragments of nucleic acids an insoluble structure called "inclusion bodies" (Makoff et al., 1990). The extraction and purification of the desired protein from inclusion bodies are laborious, expensive, and might not be commercially attractive. Moreover, although large amounts of fusion protein can be accumulated in the periplasm with reduced toxic effects to the cell, by fusing this foreign protein to the signal peptide of the *E. coli* outer membrane protein (OMPA) (MacIntyre et al., 1991), this hybrid protein is highly sensitive to proteolytic degradation by the product of *ompT* and *degP* genes (Baneyx & Georgiou, 1990). Secretion of proteins across the outer membrane into the culture medium is unusual in the Gram negative bacterium *E.coli* (Hirst, 1991). In contrast, the Gram positive bacterium *Bacillus subtilis* is able to secrete proteins into the culture medium. However, production of heterologous proteins by *B.subtilis* may be compromised by the simultaneous production of at least seven extracellular proteases (Harwood, 1992).

The yeast *Saccharomyces cerevisiae* is also an attractive host for expression of heterologous genes and protein secretion. Transcriptional regulation in this yeast has been shown to be similar to that in mammalian cells (reviewed by Bitter, 1986 and 1988). *S.cerevisiae* can perform glycosylation, acetylation, phosphorylation,

disulfide bond formation, removal of methionine at the N-terminus and proper folding, rendering biologically active products (van Brunt, 1986; Thim et al., 1989; Vlasuk et al., 1986; Green et al., 1986b; Ichikawa et al., 1989; Bourbonnais et al., 1991). This yeast is able to carry out either O-linked or N-linked glycosylation (Byrd et al., 1982; Kingsman et al., 1987; Innis et al., 1985). Furthermore, as *S.cerevisiae* secretes less than 5 % of its protein into the culture medium, a high degree of product purity could be obtained by the efficient secretion of a foreign protein using, for example, the α -factor encoding gene sequences for extracellular protein targeting (Bitter et al., 1984; Brake et al., 1984; Miyajima et al., 1985). Moreover, the scale-up fermentation process is well developed for yeast and expression systems are available (Fieschko et al., 1986; Buckholz & Gleeson, 1991). However, the yield of secreted proteins is still low. The intracellular accumulation of proteins can lead to toxic effects on the host cell and result in possible errors in the processing of the expressed foreign protein (Brake et al., 1984; Hitzeman et al., 1981; Kingsman et al., 1987). Furthermore, the yeast *S.cerevisiae* might not be the appropriate host for expression of foreign glycoproteins which requires the addition of complex carbohydrate side-chains containing sugar residues other than mannose. For instance, mammalian and viral

glycoproteins expressed in *S.cerevisiae* have been reported to have a tendency to have a higher level of mannose glycosylation (Meyhack et al., 1989; Buckholz & Gleeson, 1991; Cregg et al., 1989; Jagadish et al., 1990). Such altered glycosylation in recombinant human hormones might lead to abnormal biological activity or adverse immunological response, which are undesirable for pharmaceutical applications. The recombinant subunit vaccines also require the production of authentic virus glycoproteins capable of inducing virus neutralizing antibodies. Carbohydrate structures have been shown to be important antigenic determinants (Feizi & Childs, 1987). Thus, changes in carbohydrate structure of viral glycoproteins might induce different degree of immunogenicity. Therefore, vaccines produced with non-authentic viral glycoproteins could confer no protection against the respective viral infection.

In consequence, alternative host cells which have a high level of transcription and translation and the ability to carry out post-translational modification and secretion have been considered. A variety of prokaryotic and eukaryotic genes have been expressed in organisms ranging from bacteria and yeast to sheep. The host cell has not been an obstacle in choosing the expression system. However, the characteristics of the protein to be produced (for instance, requirements for secretion

and glycosylation) and in what application the protein is going to be used, added to economic considerations, have dictated the choice of host-vector system.

In this regard, the filamentous fungi have gained consideration as a host for heterologous expression and protein secretion for several reasons. First, and most importantly, certain species are capable of secreting large amounts of native proteins (up to 30 g/l), for example, glucoamylase secreted by *Aspergillus niger*; amylase by *Aspergillus oryzae* and cellobiohydrolase by *Trichoderma reesei*. Second, native enzymes produced by many species are generally regarded as safe for human consumption by the regulatory authorities (Barbesgaard et al., 1992). Third, gene mediated transformation systems are available for many species. Fourth, filamentous fungi perform both N- and O-linked protein glycosylation in a manner similar to that in higher eukaryotes at least in terms of the content of mannose residues (Ward et al., 1992a; Saunders et al., 1989; Salovuori et al., 1987). Fifth, large scale fermentation and down-stream processing procedures are well established (Gwynne, 1992; Dunn-Coleman et al., 1992). Finally, filamentous fungi represent alternative hosts for the commercial production of those proteins impeded by process patents involving other organisms.

1.2 Gene transfer systems for filamentous fungi

Although transformation using lithium acetate treatment of conidia (Allisson et al., 1992), electroporation (Thomas & Kenerley, 1989) and tungsten microprojectiles (Bailey et al., 1993) have been reported for filamentous fungi, DNA uptake by protoplasts is by far the most widely used method for introducing free DNA into fungal cells. The procedure involves three steps. (1) Formation of protoplasts in medium containing an osmotic stabilizer. This is achieved by removal of the cell wall with suitable lytic enzymes in the presence of MgSO_4 , which facilitates the separation of the protoplasts from mycelial debris. (2) DNA uptake into protoplasts mediated by polyethylene glycol (PEG) in the presence of Ca^{++} . The former induces fusion of protoplasts that, in turn, helps DNA trapping. (3) Regeneration of protoplasts and selection of transformants by plating the DNA-treated cells on agar medium, that is selective for the desired transformant and contains an osmotic stabilizer. There are a variety of selection systems based on complementation of specific auxotrophic mutations (Table 1.1). These systems require an appropriate, cloned, prototrophic gene as a nutritional marker and a stable, corresponding mutant strain. Mutants have been obtained by using efficient, direct selection methods, such as selection

Table 1.1 Transformation selection system using nutritional markers

Marker	Selection	Species	References
<i>acuD,E</i>	acetate utilisation	<i>Aspergillus nidulans</i>	Buxton <i>et al.</i> , 1989 Turner <i>et al.</i> , 1985
<i>argB</i>	arginine prototrophy	<i>Aspergillus awamori</i> <i>Aspergillus nidulans</i> <i>Aspergillus niger</i> <i>Aspergillus oryzae</i> <i>Aspergillus terreus</i> <i>Gibberella fujikuroi</i> <i>Trichoderma reesei</i>	Ward, 1989a John & Peberdy, 1984 Buxton <i>et al.</i> , 1985 Gomi <i>et al.</i> , 1987 Hahn & Batt, 1988 Ventura & Raman, 1991 Bruckner <i>et al.</i> , 1992 Penttilä <i>et al.</i> , 1987
<i>amdS</i>	acetamide utilisation	<i>Aspergillus terreus</i> <i>Aspergillus nidulans</i> <i>Aspergillus niger</i> <i>Aspergillus oryzae</i> <i>Cochliobolus heterostrophus</i> <i>C. lindemuthianum</i> <i>Penicillium chrysogenum</i> <i>Trichoderma reesei</i>	Davis <i>et al.</i> , 1988 Tilburn <i>et al.</i> , 1983 Kelly & Hynes, 1985 Gomi <i>et al.</i> , 1991 Turgeon <i>et al.</i> , 1985 Rodriguez & Yoder, 1987 Kolar <i>et al.</i> , 1988 Paloheimo <i>et al.</i> , 1992
<i>invA</i>	invertase prototrophy	<i>Neurospora crassa</i> <i>Aspergillus niger</i>	Caru <i>et al.</i> , 1989 Berges <i>et al.</i> , 1993
<i>leuA</i>	leucine prototrophy	<i>Mucor circinelloides</i> <i>Rhizopus niveus</i>	Roncero <i>et al.</i> , 1989 Sakaguchi <i>et al.</i> , 1992
<i>metA</i>	methionine prototrophy	<i>Aspergillus oryzae</i>	Iimura <i>et al.</i> , 1987
<i>niaD</i>	nitrate utilisation	<i>Aphanocladium album</i> <i>Aspergillus flavus</i> <i>Aspergillus nidulans</i> <i>Aspergillus niger</i> <i>Aspergillus oryzae</i> <i>Aspergillus parasiticus</i> <i>Beauveria bassiana</i> <i>Colletotrichum lindemuthianum</i> <i>Gibberella fujikuroi</i> <i>Fusarium graminearum</i> <i>Fusarium oxysporum</i> <i>Nectria haematococca</i> <i>Neurospora crassa</i> <i>Penicillium caseicolum</i> <i>Penicillium chrysogenum</i> <i>Pyricularia oryzae</i>	Daboussi <i>et al.</i> , 1989 Chevalet <i>et al.</i> , 1992 Johnstone <i>et al.</i> , 1985 Unkles <i>et al.</i> , 1989a Unkles <i>et al.</i> , 1989b Chang <i>et al.</i> , 1992 Daboussi <i>et al.</i> , 1989 Daboussi <i>et al.</i> , 1989 Sanchez-Fernandez <i>et al.</i> , 1991 Sims <i>et al.</i> , 1992 Malardier <i>et al.</i> , 1989 Daboussi <i>et al.</i> , 1989 Tomsett & Garrett, 1980 Daboussi <i>et al.</i> , 1989 Whitehead <i>et al.</i> , 1989 Daboussi <i>et al.</i> , 1989
<i>pyrG, pyr4</i>	uridine prototrophy	<i>Aspergillus awamori</i> <i>Aspergillus flavus</i> <i>Aspergillus nidulans</i> <i>Aspergillus niger</i> <i>Aspergillus oryzae</i> <i>Mucor circinelloides</i> <i>Neurospora crassa</i> <i>Penicillium chrysogenum</i> <i>Phanerochaete chrysosporium</i> <i>Podospora anserina</i> <i>Trichoderma reesei</i>	Ward, 1989b Woloshuk <i>et al.</i> , 1989 Ballance <i>et al.</i> , 1983 Van Hartingsveldt <i>et al.</i> , 1987 Mattern <i>et al.</i> , 1987 Benito <i>et al.</i> , 1992 Buxton & Radford, 1984 Beri & Turner, 1987 Alic <i>et al.</i> , 1992 Perrot <i>et al.</i> , 1987 Smith <i>et al.</i> , 1991
<i>pacA</i>	phospho-esterase prototrophy	<i>Aspergillus nidulans</i>	MacRae <i>et al.</i> , 1988

Table 1.1 Continuation

<i>pmA</i>	proline prototrophy	<i>Aspergillus nidulans</i>	Davis <i>et al.</i> , 1988 Turnbull <i>et al.</i> , 1989
<i>qufE</i>	quinic acid utilisation	<i>Aspergillus nidulans</i>	Streatfield <i>et al.</i> , 1993 Da Silva, 1986
<i>qa-2</i>		<i>Neurospora crassa</i>	Case <i>et al.</i> , 1982
<i>sC</i>	sulphate utilisation	<i>Aspergillus nidulans</i>	Buxton <i>et al.</i> , 1989 Borges <i>et al.</i> , 1992
<i>suc1</i>	sucrose utilisation	<i>Podospora anserina</i> <i>Trichoderma hamatum</i> <i>Trichoderma reesei</i>	Barreau <i>et al.</i> , 1992 Barreau <i>et al.</i> , 1992 Barreau <i>et al.</i> , 1992
<i>trpC</i>	tryptophan prototrophy	<i>Aspergillus nidulans</i> <i>Coprinus cinereus</i> <i>Neurospora crassa</i> <i>Penicillium chrysogenum</i>	Yelton <i>et al.</i> , 1984 Binninger <i>et al.</i> , 1987 Challen <i>et al.</i> , 1992 Case <i>et al.</i> , 1979 Sanchez <i>et al.</i> , 1987

for chlorate resistance for nitrate reductase deficient mutants (Cove, 1979) and selection by selenate for adenosine triphosphate sulfurylase mutants (Buxton et al., 1989). Furthermore, a nutritional marker does not seem to be stringently species-specific thus, for example, the *A.nidulans niaD* gene can be used in other filamentous fungi harbouring the *niaD*⁻ mutation in heterologous transformation (Daboussi et al., 1989). However, specific auxotrophic mutants of industrial strains can be difficult to obtain. An alternative is to use a dominant mutant gene as selective marker, such as those which confer drug resistance (Table 1.2) (for a review see Fincham, 1989 and Turner & Ballance, 1985). A nutritional marker such as the *Aspergillus niger suc1* gene, which if *Aspergillus* were to be used as the transformation recipient strain, would require an invertase-minus mutant unable to grow on sucrose, can also be used as a dominant selective marker in *Trichoderma reesei* transformation, since this species naturally lacks invertase activity (Berges et al., 1993). Furthermore, gene disruptions can be obtained by transforming a wild type strain with a plasmid containing part of the gene of interest. This technique generates auxotrophic mutants containing the gene disrupted by the plasmid sequences (Fincham, 1989).

Table 1.2 Transformation selection system using dominant markers

Marker	Selection (resistance to)	Species	References
<i>benA</i>	benomyl	<i>Aspergillus nidulans</i> <i>Aspergillus niger</i> <i>Cryphonectria parasitica</i> <i>Gaeumannomyces graminis</i> <i>Neurospora crassa</i>	May <i>et al.</i> , 1985 Ferreira & Bonatelli, 1992 Razanamparany <i>et al.</i> , 1992 Henson <i>et al.</i> , 1988 Orbach <i>et al.</i> , 1986
Blasticidin S	blastocidin	<i>Rhizopus niveus</i>	Yanai <i>et al.</i> , 1991
<i>bleR</i>	bleomycin pheomycin neomycin	<i>Aspergillus nidulans</i> <i>Aspergillus niger</i> <i>Cephalosporium acremonium</i> <i>Cochliobolus lunatus</i> <i>Neurospora crassa</i> <i>Parasitella parasitica</i> <i>Penicillium chrysogenum</i> <i>Penicillium roqueforti</i> <i>Tolypocladium geodes</i> <i>Trichosporium cutaneum</i>	Austin <i>et al.</i> , 1990 Finkelstein <i>et al.</i> , 1989 Mathison <i>et al.</i> , 1993 Rozman & Komel, 1992 Austin <i>et al.</i> , 1990 Kellner <i>et al.</i> , 1993 Kolar <i>et al.</i> , 1988 Durand <i>et al.</i> , 1990 Calmels <i>et al.</i> , 1991 Glumoff <i>et al.</i> , 1989
G418	G418 kanamycin	<i>Absidia glauca</i> <i>Ashbya gossypii</i> <i>Cephalosporium acremonium</i> <i>Mucor circinelloides</i> <i>Neurospora crassa</i> <i>Phanerochaete chrysosporium</i> <i>Phycomyces blakesleeanus</i> <i>Rhizopus niveus</i> <i>Ustilago maydis</i>	Sakaguchi <i>et al.</i> , 1992 Wright & Philippsen, 1991 Penalva <i>et al.</i> , 1985 Sakaguchi <i>et al.</i> , 1992 Bull & Wootton, 1984 Akileswaran <i>et al.</i> , 1993 Revuelta & Jayaram 1986 Sakaguchi <i>et al.</i> , 1992 Suarez & Eslava, 1988
<i>hph</i>	hygromycin	<i>Acremonium chrysogenum</i> <i>Aspergillus fumigatus</i> <i>Aspergillus nidulans</i> <i>Aspergillus niger</i> <i>Aspergillus terreus</i> <i>Cephalosporium acremonium</i> <i>Colletotrichum lindemuthianum</i> <i>Cryphonectria parasitica</i> <i>Gibberella fujikuroi</i> <i>Gibberella pulicaris</i> <i>Gliocladium roseum</i> <i>Gliocladium virens</i> <i>Glomerella cingulata</i> <i>Hebeloma cylindroporum</i> <i>Humicola grisea</i> <i>Fusarium oxysporum</i> <i>Fusarium solani</i> <i>Fulvia fulva</i> <i>Laccaria laccata</i> <i>Leptosphaeria maculans</i> <i>Magnaporthe grisea</i> <i>Nectria haematococca</i> <i>Neurospora crassa</i> <i>Penicillium citrinum</i> <i>Penicillium roqueforti</i> <i>Phytophthora spp</i> <i>Podospora anserina</i> Steril red fungus <i>Trichoderma hamatum</i> <i>Trichoderma harzianum</i> <i>Trichoderma viridae</i> <i>Ustilago maydis</i>	Smith <i>et al.</i> , 1992 Tang <i>et al.</i> , 1992 Cullen <i>et al.</i> , 1987 Punt <i>et al.</i> , 1987 Ventura & Raman, 1991 Queener <i>et al.</i> , 1985 Rodriguez & Yoder, 1987 Choi <i>et al.</i> , 1993 Bruckner <i>et al.</i> , 1992 Salch & Beremand, 1993 Thomas & Kenerly, 1989 Thomas & Kenerly, 1989 Rodrigues & Yoder, 1987 Marmeisse <i>et al.</i> , 1992 Allison <i>et al.</i> , 1992 Kistler & Benny, 1988 Crowhurst <i>et al.</i> , 1992 Oliver <i>et al.</i> , 1987 Barrett <i>et al.</i> , 1990 Farman & Oliver, 1988 Tooley <i>et al.</i> , 1992 Kistler & Benny, 1992 Staben <i>et al.</i> , 1989 Nara <i>et al.</i> , 1993b Durand <i>et al.</i> , 1990 Bailey <i>et al.</i> , 1993 Ridder & Osiewacz, 1992 Seth & Fisher, 1992 Ulhoa <i>et al.</i> , 1990 Ulhoa <i>et al.</i> , 1990 Herrera-Estrella <i>et al.</i> , 1990 Wang <i>et al.</i> , 1988
<i>o/c</i>	oligomycin C	<i>Aspergillus nidulans</i> <i>Aspergillus niger</i> <i>Penicillium chrysogenum</i>	Ward <i>et al.</i> , 1988 Ward <i>et al.</i> , 1988 Bull <i>et al.</i> , 1988

Where the transforming gene can not be directly selected for, the transformation can be carried out by two vectors, one containing the selective marker and the other the expression cassette. This phenomenon, called co-transformation, is based on the high probability that if a competent cell presented simultaneously with two different kinds of DNA takes up one kind of DNA it will also be able to take up the other. The identification of desired transformants obtained by means of co-transformation is in two steps. First, the transformants are selected by their phenotypic change on the selective media. Second, transformant strains carrying the expression cassette sequences are identified by hybridization of their genomic DNA to radioactive DNA probe homologous to those of the expression cassette sequences.

Integration of transforming sequences into host chromosomes results in high mitotic and meiotic stability (Gems *et al.*, 1991). There are three types of integration depending upon the position on the chromosome where the transforming sequences were integrated. (1) Homologous additive integration (type I integration) results from homologous crossing over between the cloned sequence and the highly homologous sequences on the chromosome. For example, a plasmid carrying the *niaD* gene is likely to integrate into the

resident *niaD* locus. (2) Non-homologous integration (type II integration) occurs when the cloned gene has no significant homology with chromosomal sequences. This, for example, is the case where the *Neurospora crassa* *pyr4* gene lacks strong homology with the *Aspergillus* *pyrG* locus and integrates elsewhere. (3) Replacement (type III integration) is a homologous integration in which the resident mutant allele is repaired by double cross-over without integration of all the plasmid sequences (for a review Johnstone, 1985).

Autonomously replicating plasmids based on the *Saccharomyces cerevisiae* *ans-1* sequence have been used successfully in transformation of the filamentous ascomycete *Ashbya gossypii* (Wright & Philippsen, 1991). In *Aspergillus* these plasmids failed to replicate autonomously, but the transformation frequency increased, probably due to increased stability of integration (Fincham, 1989; Ballance & Turner, 1985). Recently, an *Aspergillus* autonomously replicating sequence (ARS) was identified as a long inverted repeat separated by a short region (Gems et al., 1991). However, the *Aspergillus* transformants containing replicating plasmids appear to be mitotically less stable than those where the selectable marker had integrated into the chromosome. In contrast, transforming plasmids containing ARS sequences always

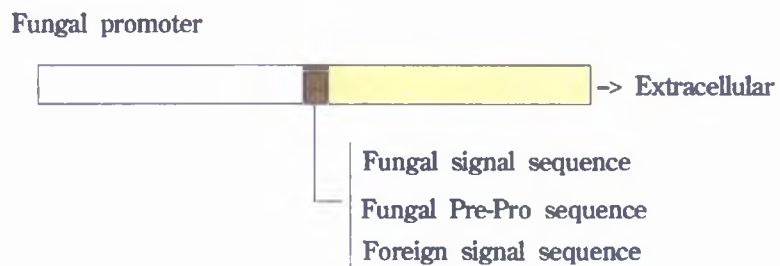
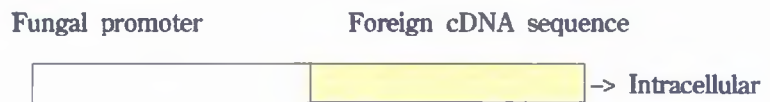
seem to replicate autonomously in *Mucor circinilloides* transformed cells (Iturriaga *et al.*, 1992; Benito *et al.*, 1992). ARS sequences from other filamentous fungi have also been identified and shown to contain the sequence TTAGGG (mentioned in Kistler & Benny, 1992), which represents the telomere-specific consensus sequence for human chromosomes.

1.3 Expression cassette

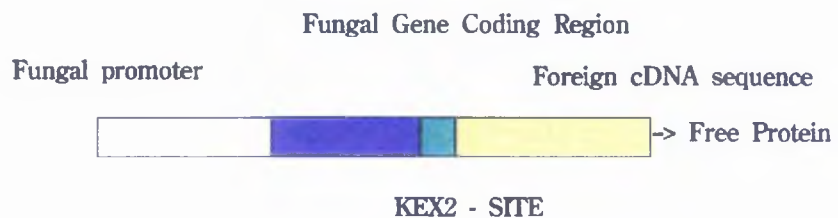
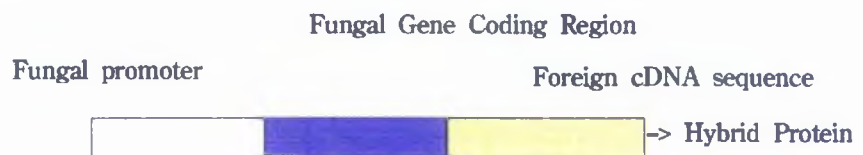
The success of heterologous expression in filamentous fungi is influenced by the expression cassette design. The basic structure of the expression cassette consists of (1) fungal transcription and translation control sequences, (2) secretion signals for expression of extracellular protein and (3) foreign protein-encoding cDNA sequence (see Figure 1.1). There are two basic designs of expression cassette. In the first, a foreign protein-encoding DNA sequence is fused directly to the fungal promoter, or through the following sequences: (1) a fungal signal sequence, (2) foreign protein signal sequence or (3) fungal protein prepro-sequences. All these sequences are included for targeting the foreign protein to the endoplasmic reticulum (see below). In the second, the N-terminus of the foreign protein is fused to the C-terminus of a

Figure 1.1 General structure of expression cassettes. Expression cassettes can harbour a foreign gene linked directly to a fungal promoter, for intracellular expression, or via different signal sequences, for extracellular expression (Panel A). Nevertheless, expression cassettes can also encode fusion proteins. The 5'end of a foreign gene can be fused direct to the 3'end of a high expressed fungal gene, generating a hybrid protein; or both genes can be separated by a KEX2-encoding DNA, which in being recognized by a KEX2-like endopeptidase allows the free form of foreign protein to be secreted (Panel B).

A



B



fungal protein yielding a fungal-foreign hybrid protein. However, the foreign protein can be obtained in a non-fused mature form, by linking the foreign and fungal protein termini through a spacer peptide containing an endopeptidase recognition site.

1.3.1 Transcription control sequences

Transcription control sequences are important elements for efficient initiation and termination of mRNA synthesis.

The Goldberg-Hogness box (TATA-box): The A-T rich canonical TATAAA can be found in most higher eukaryotic genes located at 40-100 bp upstream from the transcription start point (*tsp*) (reviewed by Gurr et al., 1987; Unkles, 1992). The TATA-box seems to be involved in fixing the start site of transcription (Paul, 1982). This TATA consensus sequence is thought to be involved in binding the TFIID component of the general transcription machinery (van Dyke & Sawadogo, 1990; Singer et al., 1990; Green, 1992).

Chambon box (CAAT-box): The conserved sequence GC^C_TCAATCT is often observed around 80 bp upstream from the *tsp* in higher eukaryotes. In filamentous fungi similar sequences are found at great distances from the

tsp, for example, such sequences in the *A.oryzae amy1* gene are found more than 300 bp away from the *tsp*. The significance of this box is unclear (reviewed by Unkles, 1992). However, recently the *A.nidulans* CCAAT-box binding factor (AnCF) has been shown to bind specifically the CCAAT sequence upstream of the *A.nidulans amdS* gene and to be involved in determining the basal level of transcription of *amdS* gene (van Heeswijck and Hynes, 1991).

Transcription start point (*tsp*): Few sequences have been recognized as being the *tsp* for filamentous fungi genes. Moreover, multiple transcription start points have been found in fungal genes (reviewed by Unkles, 1992).

Enhancer elements: Enhancers or activators are cis-acting elements in higher eukaryotes, which can function in either orientation over a considerable distances from the eukaryotic gene being transcribed (Reudelhuber, 1984; Zaman et al., 1992). Enhancers are thought to be involved in the alteration of chromatin structure and / or superhelicity to create regions of actively transcribed genes and to provide a binding site for enhancer factors which act in concert to generate enhancer activity (Khoury & Gruss, 1983; Ptashne, 1986; Nussinov, 1990). Such upstream activator sequences have been identified in some filamentous fungi promoter. For instance, the consensus TATCTA sequence located upstream

of nitrogen-regulated structural genes is recognized by the product of *Neurospora crassa nit-2* gene, a positive-acting nitrogen regulatory gene (Fu & Marzluf, 1990). Another example are the sites of action on the *A.nidulans amdS* promoter for the *trans*-acting regulatory genes *amdR* (omega amino acids induction), *amdA* (a minor acetate induction) and *facB* (acetate induction) (Kelly & Hynes, 1987; Hynes *et al.*, 1988; Hynes, 1993).

Gene regulation: Regulation of gene expression appears to involve the interaction between elements at the promoter region and regulatory proteins binding at specific upstream recognition sequences (Schaefer & Messer, 1991). There are regulatory proteins in *A.nidulans*, such as the product of the *trans*-acting regulatory genes *areA*, *nirA*, *alcR*, *amdR* and *creA*, which control the expression of an integrated subset of genes whose products all act within a particular metabolic pathway (Davis & Hynes, 1989; Hynes & Andrianopoulos, 1989; Hynes, 1993; Fu & Marzluf, 1990). The DNA-binding motifs of regulatory proteins include a highly acidic region particularly if they form amphipathic α -helices, a "zinc-finger" and S(T)PXX motifs (Drysdale *et al.*, 1993; Felenbok, 1991; Kudla *et al.*, 1990). Recently, the putative binding sequence GCGGGGC for the glucose-responsive repressor CREA protein was identified at two position upstream of the *tsp* of the *A. nidulans alcA*

gene (Hintz & Lagosky, 1993). Furthermore, evidence indicates that DNA methylation influences gene function by interfering in specific protein-DNA interactions and therefore, in the folding of the eukaryotic chromosome (Razin & Riggs, 1980). A correlation seems to exist between gene activity and DNA undermethylation and DNase I sensitivity (Saluz et al., 1991). In addition to these regulatory proteins, there are small regulating RNA species which can directly control gene expression. The regulating RNAs, named antisense RNAs, contain a complementary sequences to the target RNA, and are highly specific inhibitor of gene expression (Green et al., 1986a).

1.3.2 RNA processing and translation

mRNA processing: In higher eukaryotes, a 5' terminal "cap", $m^7G(5')ppp(5')N$, is added to the nascent nuclear pre-mRNAs. This is important in RNA stability, mRNA transport from nucleus to cytoplasm and enhancement of translation by promoting initiation complex formation (Shatkin, 1985; Nigg et al., 1991)). mRNAs that lacks this "cap" structure are thought to be protect against exonuclease attack by covalent or non-covalent interaction with proteins or by 5'-terminal secondary structure (Furuichi et al., 1977). In addition, the RNA

sequence AAUAAA signals the post-transcriptional cleavage of the primary hnRNA transcript and the subsequent addition of A residues to the free 3' end (Jackson & Standart, 1990). The poly (A) tail and 3' untranslated region are thought to function in (1) the control of mRNA stability, (2) the compartmentalization of mRNA in the cytoplasm, and (3) the control of translation by promoting interaction between 3' and 5' proximal elements of the RNA leading to reinitiation process (Jackson & Standart, 1990). It has been suggested that the mRNA degradation mechanism involves the dissociation of a poly(A)-binding protein (PABP) from the poly(A) tail which facilitates the degradation of the poly(A) tail and subsequent degradation of the body of the mRNA, catalysed by an exonuclease (3' to 5' direction) or by an endonuclease (Jackson & Standart, 1990). mRNA inactivation of hybrid sequences has been suggested to occur as result of the formation of inhibitory secondary structures. However, this can be minimized by fusing the foreign sequence to ribosome binding sites (Petersen, 1992). For instance, the expression level of hIL-6 in *E.coli* has been improved by eliminating possible mRNA secondary structure (Yasueda et al., 1990).

Start and stop codons: Almost all reported fungal genes use ATG as the translational start codon and show

preference for purine (84 %) and A (64 %) at the position -3. This position -3 is thought to modulate translation by eukaryotic ribosomes (Kozak, 1986). There is no preference in using the codons UAA (42 %), UAG (28 %) or UGA (30 %) as translational stop codon (reviewed by Unkles, 1992).

Introns: In filamentous fungi, these non-translated intervening sequences are usually short (less than 100 bp), vary in number and in position. The 5' and 3' introns boundaries consist of the nucleotide pairs GT and AG respectively which are thought to signal splicing functions (reviewed by Gurr *et al.*, 1987). The significance of introns is not clear. Different splicing of the last intron of the *A. niger glaA* gene was reported to result in two mRNA populations (Boel *et al.*, 1984). However, it is likely that the different forms of glucoamylase result of proteolytic degradation and/or different glycosylation. Introns do not occur in all fungal genes, for example, the *A.nidulans argB* gene does not have intervening sequences (Berse *et al.*, 1983).

Codon usage: Different organisms use different subsets of major codons. The main determinant for codon and anti-codon composition is the mutation bias of the DNA polymerase. However, highly expressed genes seem to be under strong selective pressure to use only a subset of codons. The correlation of the major codons and the

concentration of their cognate tRNA species is one of the selection forces operating at the level of translation. Thus, rare codons might be translated slowly because their tRNA species are in short supply so, the selection of the aminoacyl-tRNA to match these codons is rate limiting for peptide bond formation. This translational delay might be amplified by a heterologous expression cassettes integrated in high-copy-number. In addition to the sequestering effects, clusters of repeated rare codons could produce translational errors (for a review, Andersson & Kurland, 1990).

1.4 Protein targeting, transport and secretion

Several proteins are synthesized by cytoplasmic ribosomes as protein precursors, however many are found in different cellular compartments. Proteins which are transported to the endoplasmic reticulum, mitochondrion, plastid and bacterial periplasm traverse the membrane in an unfolded or partially unfolded state (Neupert et al, 1990; Bhaya & Grossman, 1991; Ellis & van der Vies, 1991). This unfolded state is facilitated by proteins called molecular chaperones, which prevent incorrect interactions between parts of other molecules, but which do not impart steric information or form on the final functional structures (Ellis & van der Vies, 1991).

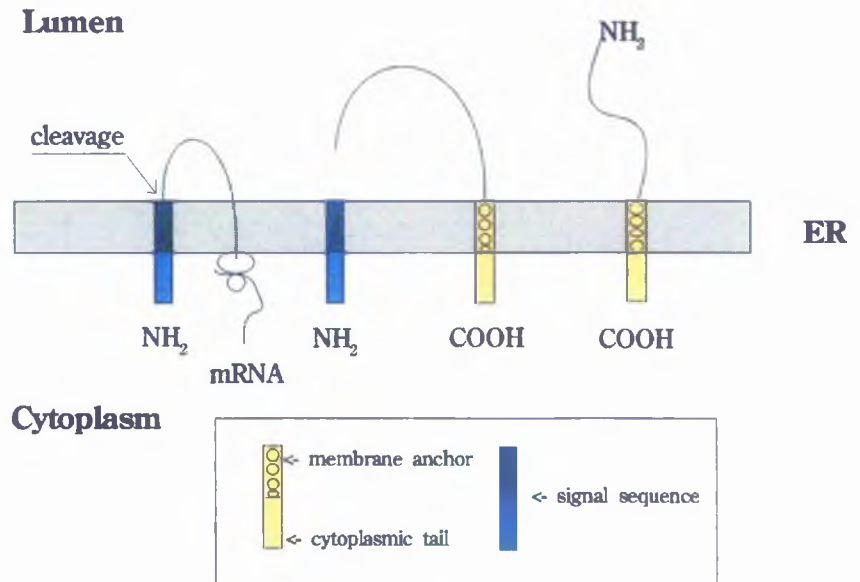
Protein translocation across the ER membrane is an essential step in the biosynthesis of many classes of proteins, including secretory proteins, plasma membrane proteins, ER resident proteins and lysosomal proteins. Proteins targeted to the ER of eukaryotic cells contain an N-terminal signal sequence (SS) of 15 to 30 residues. This sequence has a positively charged N terminus, a central hydrophobic region and a C-terminal region containing predominantly polar residues. The SS has a high propensity to adopt an α -helical conformation in bilayers or micelles and have high affinity for lipid membranes and an ability to insert into the hydrocarbon region of a lipid bilayer (Gierasch *et al.*, 1992; Kruijff, 1991). A molecular chaperone recognizes the SS secondary structure, therefore, certain chaperones can recognize different SS, as well as, two different chaperones can bind the same peptide when found in different conformations (Gierasch *et al.*, 1992).

Co-translational translocation: The transport of a protein across the rough ER begins with the recognition of the SS by the signal recognition particle (SRP) as the nascent polypeptide emerges from the large ribosomal subunit. The SRP consists of a short RNA molecule and six constituent proteins. The translocation process can be summarised in five steps. First, the SRP binds to the SS forming a tight-binding complex and arrests

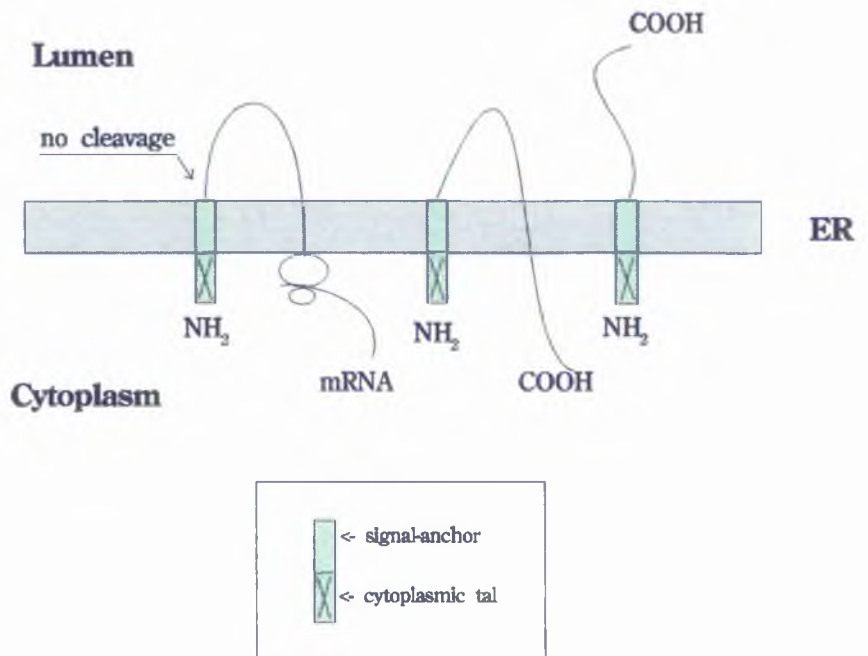
elongation. Second, the SRP binds the complex to the SRP receptor on the membrane. Third, the SS interacts with an integral membrane protein, the SS receptor. Fourth, the elongation arrest is released and the ribosome becomes bound to the two ER integral glycoproteins ribophorins I and II. Fifth, the SRP is released both from the ribosome and the SS and in the presence of GTP the protein is translocated across the membrane. The SS may or may not be cleaved by a signal peptidase in the lumen of the ER forming the mature protein (Saier et al., 1989; Rapoport et al., 1992; Gilmore, 1991). For example, the type II integral membrane glycoproteins contain an uncleaved hydrophobic N-terminal signal sequence which drives the protein into the endoplasmic reticulum (targeting function) and maintains the protein associated with the membrane (anchoring function). In contrast, the type I integral membrane glycoproteins are targeted by a cleavable N-terminal signal sequence and anchored by C-terminal stop-transfer region. Thus, the orientation adopted is external N-terminus and C-terminus in the cytoplasm. Finally, the type III membrane glycoproteins contain an uncleaved signal sequence as type II protein, but the final orientation is as type I protein (Figure 1.2) (Shaw et al., 1988; Parks & Lamb, 1991). Another alternative mode of proteins translocation involves a post-translational ATP-hydrolysis-dependent pathway, and was observed in

Figure 1.2 A model for the insertion and orientation of type I and type II glycoproteins into the ER. Signal sequences are inserted into the ER as loops or helical structures, with the N-terminus remaining on the cytoplasmic side. The nascent polypeptide is then translocated across the membrane and appears as loop structure in the lumen of the ER. In type I protein, the signal sequence is cleaved, the N-terminus is released into the lumen of the ER and the protein is anchored via the C-terminal anchor domain. In type II protein the N-terminal signal-anchorage domain remains uncleaved and anchors the protein in the ER leaving the C-terminus free in the lumen (Adapted from Shaw *et al.*, 1988).

A Type 1



B Type 2



proteins of low molecular weight in mammalian cells (Gilmore, 1991).

Protein trafficking: The export of proteins from the ER occurs by a bulk-flow mechanism. However, in order to identify resident proteins from those which must continue along the secretory pathway, a complex sorting mechanism begins in the ER. Some retention signals have been recognized. Both cytoplasmic Lys-(X)-Lys-X-X and Lys-Lys-X-X consensus signals are thought to mediate the retention of endogenous ER transmembrane proteins. While the ER luminal proteins contain a negatively charged region near the C terminus of the polypeptide and a carboxyterminal tetrapeptide consensus sequence, Lys-Asp-Glu-Leu (KDEL) in mammalian cells and His-Asp-Glu-Leu (HDEL) in *S.cerevisiae* (reviewed by Sabatini et al., 1991). The resident Golgi protein TGN38/41 is a transmembrane protein in which localization to the trans Golgi network (TGN) is mediated by its carboxy-terminal cytoplasmic tail, which contains an 11 amino acid tyrosine-based motif (Stanley & Howell, 1993; Hobman, 1993). Other signals are a mannose-6-phosphate signal for lysosomal enzymes and a tight-turn containing aromatic residues (conformational motif) for endocytosis (Sabatini et al., 1991; Vaux & Fuller, 1992; Bomsel & Mostov, 1991). The intracellular vesicular transport requires ATP and NSF protein (N-ethylmaleimide-

sensitive fusion protein). This protein acts as a molecular chaperone similar to the way that the products of *sec* genes are involved in vesicular transport in the yeast *S.cerevisiae* (Tai et al., 1992; Rexach et al., 1992; Kaiser & Schekman, 1990; Newman et al., 1990). Moreover, GTP-binding proteins may participate in vesicle formation, vesicle docking and fusion of the vesicle membrane with that of the acceptor compartment (Clary et al., 1990; Sabatini et al., 1991).

Post-translational modification: A large number of resident soluble ER proteins govern numerous post-translational modifications of proteins including cleavage of the SS, folding, disulfide-bond formation, glycosylation and oligomerization (reviewed by Vaux & Fuller, 1992). Shortly after synthesis, many translocated proteins become associated with GRP78-Bip resident ER protein, which prevents premature folding of newly synthesized proteins (Ng et al., 1989, 1990). When proteins reach their folded conformation just prior to oligomerization, dissociate from GRP78-Bip protein (Hendershot et al., 1988). In contrast, misfolded molecules are retained inside the ER by a stable interaction with GRP78-Bip for refolding or degradation (Ng et al., 1992). Correct protein folding is facilitated by the acquisition of N-glycans. There are two major groups of glycoprotein glycans, N-glycans,

which are linked to the Asn residues of polypeptides, and O-glycans linked to Ser or Thr residues. N-glycans are involved in (1) correct folding, (2) solubility and stability of the conformation of glycoproteins, (3) modulation of biological activity, (4) contributing to efficient transport and (5) protection from proteolytic degradation. However, the significance of protein O-glycosylation is not clear (Furukawa & Kobata, 1992; Elbein, 1991; Taylor & Wall, 1988; Gallagher et al., 1992; Goochee & Monica, 1990; Feizi & Childs, 1987). The addition of core glycosylation in the endoplasmic reticulum is the same in all eukaryotic cells. However, in the Golgi compartment of mammalian cells, key mannose sugars are removed from this core glycosylation chain and further addition of outer chain carbohydrates occurs. The type of processing depends upon the function the protein will have. Secretory proteins and plasma-membrane proteins have most of their mannose units removed, N-acetyl-glucosamine units added, and finally galactose and sialic acid added (Rothman, 1985). Based on the addition of outer chain structures to the common core region of N-glycans ($\text{Man}\alpha 1,6(\text{Man}\alpha 1,3) \text{Man}\beta 1, 4\text{-GlcNAc}\beta 1,4\text{-GlcNAc}$), there are the high-mannose, the complex and the hybrid types of oligosaccharides. The type of glycosylation seems to be related to the species, tissue or organ from which the cells are derived. Furthermore, glycoproteins produced by the same

cells may have different glycans which might be a result of the interference of the polypeptide structures around the glycosylation sites, denying access to one or more enzymes involved in the glycosylation process (Furukawa & Kobata, 1992). Thus, for example, a mammalian protein expressed in a fungal host might differ from the native protein regarding glycosylation pattern.

1.5 The KEX2- motif encoding DNA

Many secreted proteins are synthesized as precursors, which are processed to mature forms by limited proteolysis during their transport through the secretory pathway. For instance, *S.cerevisiae* mating α -factor consists of an N-terminal hydrophobic leader sequence, a pro-segment containing three consensus sites for addition of Asn-linked carbohydrate, and a C-terminal region consisting of repeats of the mature α -factor sequence. There are 13 aa residues in each repeat which are separated from each other by spacer regions (Fuller et al., 1988; Dmochowska et al., 1987). Three enzymes, products of the *kex2*, *kex1* and *ste13* genes, are involved in α -factor processing (Mizuno et al., 1989; Zhu et al., 1992; Cooper & Bussey, 1989; Brennan et al, 1990; Tanguy-Rougeau et al, 1988). The *S.cerevisiae* KEX2-protein, the product of the *kex2* gene, was

identified as a membrane bound protein resident in the later Golgi compartment (Redding *et al.*, 1991) and was shown to have hydrolytic specificity towards the carboxyl side of paired basic residues (Mizuno *et al.*, 1989; Thomas *et al.*, 1988). The three-dimensional structure of the precursor seems also to be important for the processing mechanism, since pairs of basic residues occur in precursor proteins that are not cleaved (Schwartz, 1986). Similar endoproteolytic processing of protein precursors at dibasic sites by a KEX2-like endoprotease has been suggested to occur in all eukaryotes (Barr, 1991; Benjannet *et al.*, 1991; Brennan *et al.*, 1990; Contreras *et al.*, 1991). Consequently, DNA sequence encoding a pair of basic residues (Lys-Arg or Arg-Arg) has been included in the structure of expression cassettes between the fungal DNA sequences and the heterologous protein encoding DNA sequences, in an attempt to obtain correctly processed, non-fused, mature proteins.

II GENES USED FOR PROMOTER ACTIVITY IN FILAMENTOUS FUNGI

Inducibility and strength are selection criteria for promoter activity in heterologous gene expression (see Table 1.3 and Table 1.4). The advantage of

Table 1.3 Inducible fungal promoters used for heterologous expression

Promoter	Gene	Species	Inductor
<i>adhA</i>	alcohol dehydrogenase	<i>Aspergillus niger</i>	ethanol
<i>alcA</i>	alcohol dehydrogenase	<i>Aspergillus nidulans</i>	ethanol
<i>alcA</i>	alcohol dehydrogenase	<i>Aspergillus niger</i>	ethanol
<i>alcC</i>	alcohol dehydrogenase	<i>Aspergillus nidulans</i>	ethanol
<i>amdS</i>	acetamidase	<i>Aspergillus nidulans</i>	acetamide w-amino acids acetate
<i>amyA,B</i>	alpha-amylase	<i>Aspergillus niger</i>	starch and
<i>amy3</i>	alpha-amylase	<i>Aspergillus oryzae</i>	maltodextrin
<i>aphA</i>	acid phosphatase	<i>Aspergillus niger</i>	'pH signals'
<i>cbh1</i>	cellobiohydrolase	<i>Trichoderma reesei</i>	cellulose
<i>gla A</i>	glucoamylase	<i>Aspergillus awamori</i>	starch and
<i>gla A</i>	glucoamylase	<i>Aspergillus niger</i>	maltodextrin
<i>mprA</i>	aspartyl protease	<i>Mucor miehei</i>	'pH signals'
<i>pacA</i>	phosphate-repressible acid phosphatase	<i>Aspergillus niger</i>	phosphate and 'pH signals'
<i>pcbC</i>	isopenicillium N synthetase	<i>Penicillium chrysogenum</i>	?
<i>pe</i>	pectin esterase	<i>Aspergillus niger</i>	?
<i>pelA</i>	pectin lyase	<i>Aspergillus niger</i>	?
<i>pgkA</i>	phosphoglycerate kinase	<i>A. nidulans</i>	oxygen

? Information not provided

For references, see van den Hondel *et al.*, 1991 and Arst, 1993.

inducible promoters is that they can be used to control transcription, therefore protein synthesis can be restricted during the fermentation process, avoiding the possible toxic effect on the cell growth as a result of heterologous protein over-production. A fungal promoter is a stringent requirement for the expression of foreign proteins in fungal systems. For instance, the expression of a bacterial aspartase in *A.nidulans* has been possible by placing the *aspA* gene under the control of the constitutive *A.nidulans* *gpdA* promoter and the *trpC* terminator (Hunter et al., 1992). Another example is where the *A.niger* *glaA* and *A.nidulans* *alcA* promoters have permitted the expression of the human interferon α -2 protein in *A.nidulans* (Gwynne et al., 1987). Several promoters have also been fused to bacterial *hph* (hygromycin) and *ble* (bleomycin) genes to allow these genes to be used as dominant selectable markers in fungal systems (Table 1.5) (Churchill et al., 1990). Another aspect of promoter activity is related to the replacement of a fungal promoter by another based on the above criteria, in order to improve the production of commercially important fungal protein both in homologous or heterologous expression system. For instance, the *A.niger* *glaA* promoter has been replaced by the strong constitutive *A.nidulans* *gpdA* promoter to control the glucoamylase expression in *A.niger* (Van den Hondel et al., 1991). Furthermore, heterologous expression of

Table 1.4 Constitutive fungal promoters used for heterologous expression

Promoter	Gene	Species
<i>gpdA</i>	glyceraldehyde-3-phosphate dehydrogenase	<i>Aspergillus nidulans</i>
<i>gpdA</i>	glyceraldehyde-3-phosphate dehydrogenase	<i>Aspergillus niger</i>
<i>oliC</i>	ATPase subunit 9	<i>Aspergillus nidulans</i>
<i>oliC</i>	ATPase subunit 9	<i>Aspergillus niger</i>
<i>oliC</i>	ATPase subunit 9	<i>Neurospora crassa</i>
<i>triA</i>	triose-phosphate isomerase	<i>Aspergillus nidulans</i>

For references, see van den Hondel *et al.*, 1991.

Table 1.5 Promoters used to drive dominant selective marker encoding gene

Marker	Selection	Source	Promoter	References
<i>E.coli hph</i>	Hygromycin	<i>Acremonium chrysogenum</i>	<i>pcbC</i>	Smith <i>et al.</i> , 1992
		<i>Aspergillus flavus</i>	<i>trpC</i>	Salch & Beremand,1993
		<i>Aspergillus nidulans</i>	<i>gpdA</i>	Punt <i>et al.</i> , 1987
			<i>pgk</i>	Nara <i>et al.</i> , 1993a
			<i>trpC</i>	Choi <i>et al.</i> , 1993
		<i>Bumia lactucae</i>	<i>ham34</i>	Judelson <i>et al.</i> , 1993
		<i>Penicillium citrinum</i>	<i>pgk</i>	Nara <i>et al.</i> , 1993b
		<i>Podospore anserina</i>	<i>gpdA</i>	Ridder & Oslewacz,1992
		<i>Trichoderma harzianum</i>	<i>amyB</i>	Lorito <i>et al.</i> , 1993
		<i>Ustilago maydis</i>	<i>hsp70</i>	Bailey <i>et al.</i> , 1993
<i>E.coli ble</i>	bleomycin	<i>Aspergillus nidulans</i>	<i>gpdA</i>	Glumoff <i>et al.</i> , 1989
			<i>oliC</i>	Allison <i>et al.</i> , 1992
		<i>Neurospora crassa</i>	<i>amdS</i>	Kolar <i>et al.</i> , 1988
			<i>am</i>	Austin <i>et al.</i> , 1990

glucoamylase (*gamP*) in *Trichoderma reesei* under the control of the *T. reesei cbh1* gene resulted in twenty-fold more glucoamylase being produced than naturally by *Hormoconis resinae* (Joutsjoki et al., 1993a,b). Several foreign proteins have been expressed under the control of *A.oryzae* amylase (*amy3*) and *A.niger* glucoamylase (*glaA*) promoters (see below).

2.1 Alpha-Amylase gene

Alpha-amylase (E.C.3.2.1.1, α -1,4-glucan-4 glucanohydrolase) is an endoenzyme which hydrolyses, randomly, α -1,4-glucosidic linkages of starch and related substrates, releasing dextrans and short oligosaccharides. Its activity is inducible by starch or maltose and regulated by exogenous glucose or low molecular weight metabolites thereof (Tanabe & Tonomura, 1954).

A wide range of organisms (Takekawa et al., 1991; Rothstein et al., 1984; Tada et al., 1989; Korman et al., 1990; Sato et al., 1986; Nakamura et al., 1986) produce amylases. Bacterial and fungal amylases hold an important place in the world market for enzymes, representing 12 % of the total sales. Alpha- amylase is utilized in the production of glucose syrup, dextrose, reducing sugars and high fructose syrup, as well as

bread-making, the production of beer, spirits, oriental food, and as a digestive aid by the pharmaceutical industry. Furthermore, alpha-amylase has been used in many industrial processes where the production of reducing sugars is an important initial step, for example, in brewing, textile and paper industries (reviewed by Lonsane & Ramesh, 1990).

The genus *Aspergillus* is the major source of fungal alpha-amylase. It was first produced by the Koji process in the manufacture of traditional Japanese food, including soya sauce, miso, sake and a semi-solid cheese-like food (Takamine, 1894). Koji is a preparation of mouldy-bran grown in semi-solid tray culture which has been inoculated by *A. oryzae* and consists of a rich source of alpha-amylase, and other enzymes (Miall, 1975).

Aspergillus oryzae has three alpha-amylase genes (*amy1*, *amy2*, *amy3*) which have high sequence homology, although, no specific *amy2* mRNA has been found (Wirsel et al., 1989). All these genes have eight introns at the same position which conform to the GT/AG rule for 5' and 3'-boundaries. The 5'-untranslated regions have a TATA box and CAAT motif at 32 and 125 bp upstream from the transcription start. *amy1* and *amy2* have a second CAAT motif at -310 position. GC boxes, probably, related with initiation of transcription are also found. The

nucleotide sequences differ in two substitutions in the *amy3* gene, codon 35 is changed from CAG to CGG and codon 151 from TTC to CTG. The main divergence is at their 3'-untranslated region downstream 70 bp from the stop codons (Wirsel et al., 1989; Tada et al., 1989). Based on the intracellular restriction fragment length polymorphisms of the α -amylase gene from *A.oryzae*, Gomi and colleagues (1990) suggested that strain variation could be responsible for divergence in the nucleotide sequence (Gomi et al., 1990). The *amy3* coding region contain a leader peptide of 21 residues with alanine at the cleavage site of the leader.

Taka-amylase A is a glycoprotein with a molecular weight of 50-55 kDa. Difference in the carbohydrate moieties induce slightly different mobilities in polyacrylamide gel electrophoresis. Four fractions were observed. Three of these fractions have a similar sugar composition; one N-glycosidic and one O-glycosidic glucosamine residue and about 5 mannose residues per molecule, while the last one has only one N-glycosidic glucosamine residue (Hase et al., 1982). Taka - amylase protein is folded into two globular domains. Domain A comprises of amino acids 1 to 380 and is related to substrate binding and catalytic function. Domain B is smaller, consisting of amino acids 381 to

478, with as yet unknown function (Matsuura et al., 1984; Ito et al., 1987).

Taka - amylase control regions have been used to express heterologous proteins in the genus *Aspergillus* (Boel et al., 1987). The structure of the expression cassette used consisted of the gene of interest linked to the promoter, terminator and polyadenylation sequences of Taka-amylase (Boel et al., 1987).

It is my intention in this study to construct efficient expression vectors using the entire alpha-amylase gene from *A.oryzae* (see Results Section 3).

2.2 Glucoamylase gene

Glucoamylase or amyloglucosidase [EC 3.2.1.3, α -(1-4), (1-6) -D-glucan glucohydrolyse] catalyses the release of D-glucose from the non-reducing ends of oligo- or polysaccharides such as maltose and starch, by hydrolysis of α -1,4-D-glucosidic linkages as well α -1,6-D-glucosidic bonds. This enzyme is very important in the glucose syrup and alcohol industry, where it is used for saccharification of starch from different sources. This represents the first step of many industrial processes which require glucose as substrate, for instance, the production of crystallized dextrose, fructose production and alcoholic fermentation.

The glucoamylase (*glaA*) gene from *A.niger* exists as a single copy in the genome (Boel *et al.*, 1984; Nunberg *et al.*, 1984). It contains a typical eukaryotic promoter with TATAA box and CAAT box motifs located at the -35 region and -100 region respectively. There are two initiation points starting at the second A in the sequence $C_G A A C_G$, resulting in a 5'-untranslated region of 68 or 44 nucleotides, respectively 30 % and 70 % of the mRNA population. Multiple initiation points where transcription starts at the second A in the above sequence have been described for highly transcribed genes of lower eukaryotes, for instance, alcohol dehydrogenase I (ADHI) (Bennetzen & Hall, 1982) and 3-phospho-glycerate kinase (PGK) (Hitzeman *et al.*, 1982) genes from *S.cerevisiae*. The *glaA* structural gene has 1,920 nucleotides encoding a protein of 640 amino acids. The first 24 amino acids are not present in the mature protein. It is suggested that a secretory signal sequence comprises the first 18 amino acids and is probably cleaved by a signal peptidase between residues Ala₁₈ and Asn₁₉, and the following six residues represent a small propeptide that is processed by a trypsin-like protease at the carboxyl site of the dibasic peptide Lsy₂₃ - Arg₂₄, forming a mature protein of 616 amino acids. However, there are two forms of glucoamylase differing in size. There is evidence that the *glaA* gene expresses the two forms of the enzyme by

differential mRNA splicing (Boel et al., 1984). The gene contains five intervening sequences. The one 169-bp intron is said to be responsible for the expression of the two forms of glucoamylase. The other four introns (A, B, C, and D) range from 55 to 75 nucleotides. All introns begin with the sequence GTNNGT and end in T/CAG. As a consequence of the differential splicing of the fifth intron, a deletion of 169bp may or may not occur between Ser₅₂₇ and Ser₅₈₃ corresponding codons, which results in a shorter mRNA with reading frame changed at the COOH- terminus (Boel et al., 1984). Thus, both forms of glucoamylase have a common NH₂-terminus, but differ in the COOH-terminus. G1 has an open reading frame of 640 amino acids while G2 contains an open reading frame of 513 amino acids. The two non-glycosylated forms of glucoamylase produced by *in vitro* translation have relative molecular weights of 71,000 and 61,000. Glycosylation, usually, adds 20 - 30 % to the molecular mass (Shibuya et al., 1990). Glycosylated forms have apparent molecular weights of 85,000 and 75,000 respectively (Berka et al., 1991). However, multiple molecular weights and amino acids compositions for the various forms of glucoamylase have been found in culture broths (Svensson et al., 1986). This heterogeneity seems to be due to proteases and/or glycosidases secreted into the culture medium (Berka et al., 1991 and references therein). However, only the

starch (Ueda, 1981) and it also hydrolyses soluble starch three times faster than the smaller G2. Glucoamylase contains three functional domains, a catalytic domain (aa 1-440), a peptide region rich in Thr and Ser residues (TS region, aa 441-512) and C-terminal domain (aa 513-616). The TS region contains a high succession of hydroxy amino acids in association with short mannoside chains and is thought to be the raw starch binding region (Hayashida et al., 1989a,b). However, it has been suggested that the TS-encoding region is only a linker between the starch binding and the catalytic domains (Evans et al., 1990).

The *A. oryzae* glucoamylase gene encodes 612 amino acids. The deduced molecular weight of non-glycosylated protein is 66 kDa (Hata et al., 1991a,b). *A.oryzae* and *A.niger* (black-koji fungal strain) glucoamylases share 67 % homology in the deduced amino acid sequence. Furthermore, genomic DNA of *A.oryzae* does not hybridize with the glucoamylase cDNA probe from *A.shirousami* (white-koji fungal strain). This evidence suggests that *A.oryzae* has a different type of glucoamylase from other *Aspergillus* species (Shibuya et al., 1990). *A.oryzae* glucoamylase differs also in its raw starch digestion ability and productivity in solid culture. The TS-region which shows partial deletion consists of only seven residues of Thr/Ser. Five highly homologous regions

among fungal glucoamylases were described. Two of these regions, in which the level of homology between *A.oryzae* and *A.niger* genes are 78 % and 94 % respectively, seem to be responsible for binding substrates and for catalytic activity (Hata et al., 1991a,b).

Expression cassettes have been constructed using glucoamylase control sequences to drive the expression of heterologous proteins in *Aspergillus* species (Contreras et al., 1991; Cullen et al., 1987; Ward et al., 1990). Two basic constructions have been employed. The one consists of the fusion of the *glaA* promoter sequences to the foreign cDNA sequences, as reported for interferon α -2 and interleukin-6 (Gwynne et al., 1987; Carrez et al., 1990). The interleukin-6 expression was influenced by the type of sequence used to link the *glaA* promoter to the interleukin-6 cDNA sequence, i.e, the *glaA* signal or propeptide sequences (Carrez et al., 1990). The other expression cassette has been generated by fusing the complete *A.niger glaA* gene to the foreign cDNA sequence, for example, calf chymosin, interleukin-6 and porcine pancreatic prothrombinase A₂ cDNA sequences (Ward, 1991; Contreras et al., 1991; Roberts et al., 1992).

III HETEROLOGOUS GENE EXPRESSION IN FILAMENTOUS FUNGI

3.1 Bacterial genes in fungi

An increasing number of fungal promoters have been studied using a reporter genes. Reporter genes consist of a chimaeric genes in which the desired promoter is fused to the coding sequence of a gene encoding a protein that is easily detected, e.g. the *E.coli uidA* (β -glucuronidase) or *lacZ* (β -galactosidase) genes (Mattern & Punt, 1988; Roberts *et al.*, 1989). Co-transformation of this vector into filamentous fungi results in the expression of these proteins which are easily detected and assayed. Consequently, this strategy permits the analysis of the control of gene expression by the promoter assayed (Table 1.6).

Another example of heterologous gene expressed in filamentous fungi is the *E.coli* heat labile enterotoxin subunit B (LTB) gene which has been expressed in *Aspergillus nidulans* under the control of the *A.nidulans amdS* promoter (Turnbull *et al.*, 1989). The highest intracellular expression level was 24 μ g of LTB per g wet weight of mycelia. The failure to detect LTB in the medium was likely to be a result of the degradation of the secreted LTB protein by the host extracellular proteases, since the absence of alpha subunit does not avoid the folding of LTB protein (Green, unpublished).

Table 1.6 Bacterial proteins produced in filamentous fungi

Host	Protein	Source	Promoter	References
<i>A.nidulans</i>	Aspartase	<i>E.coli</i>	<i>gpdA</i>	Hunter <i>et al.</i> , 1992
	B-galactosidase	<i>E.coli</i>	<i>amdS</i>	Davis <i>et al.</i> , 1988
			<i>gpdA</i>	Kolar <i>et al.</i> , 1988
			<i>ipns</i>	Perez-Esteban <i>et al.</i> , 1993
			<i>niiA</i>	Punt <i>et al.</i> , 1991
			<i>oliC</i>	Turner <i>et al.</i> , 1985
			<i>trpC</i>	van Gorcom <i>et al.</i> , 1986
	B-glucuronidase	<i>E.coli</i>	<i>gpdA</i>	van den Hondel <i>et al.</i> , 1985
			<i>niaD</i>	Roberts <i>et al.</i> , 1989
	Chroraphenicol acetyl transferase	<i>E.coli</i>	<i>CaMV35S</i>	Punt <i>et al.</i> , 1991
	Endogluconase	<i>C.fine</i>		Riddell <i>et al.</i> , 1992
	Enterotoxin subunit B	<i>E.coli</i>	<i>alcA</i>	Gwynne <i>et al.</i> , 1987
			<i>glaA</i>	Gwynne <i>et al.</i> , 1987
			<i>amdS</i>	Turnbull <i>et al.</i> , 1989
<i>A.niger</i>	B-galactosidase	<i>E.coli</i>	<i>amdS</i>	Davis <i>et al.</i> , 1988
			<i>gpdA</i>	van Gorcon <i>et al.</i> , 1986
	B-glucuronidase	<i>E.coli</i>	<i>trpC</i>	van Gorcon <i>et al.</i> , 1986
			<i>gpdA</i>	Roberts <i>et al.</i> , 1989
<i>A.oryzae</i>	B-galactosidase	<i>E.coli</i>	<i>gpdA</i>	Ruiter-Jacobs <i>et al.</i> , 1989
	B-glucuronidase	<i>E.coli</i>	<i>amy</i>	Tada <i>et al.</i> , 1991
			<i>gpdA</i>	Ruiter-Jacobs <i>et al.</i> , 1989
<i>A.terreus</i>	B-galactosidase	<i>E.coli</i>	<i>amdS</i>	Davis <i>et al.</i> , 1988
<i>Fulvia fulva</i>	B-glucuronidase	<i>E.coli</i>	<i>gpdA</i>	Roberts <i>et al.</i> , 1989
<i>P.chrysogenum</i>	B-galactosidase	<i>E.coli</i>	<i>gpdA</i>	Kolar <i>et al.</i> , 1988
	Expandase	<i>S.clavuligerus</i>	<i>pcbC</i>	van den Hondel <i>et al.</i> , 1991
<i>Phytophthora ssp</i>	B-glucuronidase	<i>E.coli</i>	<i>CaMV35S</i>	Bailey <i>et al.</i> , 1993
<i>Rhizopus niveus</i>	B-galactosidase	<i>E.coli</i>	<i>RNAPII</i>	Yanai <i>et al.</i> , 1991
<i>T.geodes</i>	B-galactosidase	<i>E.coli</i>	TR1	Baron <i>et al.</i> , 1992
	Phe binding protein	<i>S. hindustanicus</i>	<i>gpdA</i>	Calmels <i>et al.</i> , 1991
<i>T.reesei</i>	B-galactosidase	<i>E.coli</i>	<i>gpd1</i>	Penttila <i>et al.</i> , 1987

The *Streptoalloteichus hindustanus* phleomycin binding protein (SH) has been successfully expressed in *Tolypocladium geodes*. Surprisingly, high expression levels of the secreted protein, up to 1.5 g/l, have been obtained (Calmels et al., 1991). This high level was suggested to be as the result of the lack of rare dipeptides on the SH protein sequence, which enables the protein to escape proteolytic digestion. Moreover, the recipient strain used was a hypersecretory mutant deficient in extracellular proteases.

3.2 Fungal gene in fungi

The main aims of heterologous expression in fungi are related to strain improvements (see Table 1.7). For example, *A.oryzae*, the most common fungus employed in the fermentation of sake in Japan, has little raw starch degradative activity (RSD), which is associated with yield and quality in alcoholic beverage fermentation processes. Therefore, to improve its RSD activity and glucoamylase activity, the glucoamylase gene from *A.shirousami* was introduced into *A.oryzae* (Shibuya et al., 1990). As a result, a transformant strain had six-fold higher glucoamylase activity and two-fold higher RSD activity than the host strain when it grew in Czapek-Dox minimal medium containing 2 % maltose as a

Table 1.7 Heterologous fungal protein produced in filamentous fungi

Host	Protein (Source)	Promoter	Yield (mg/l)	References
<i>A.awamori</i>	Alpha amylase (<i>A.oryzae</i>)	<i>amy</i>	1500	van den Hondel <i>et al.</i> , 1991
	Aspartyl protease (<i>M.miehei</i>)	?	3000	van den Hondel <i>et al.</i> , 1991
	Galactose oxidase (<i>Dactylium dendroides</i>)	<i>gaoA</i>	?	Stevens <i>et al.</i> , 1992
	Glucoamylase (<i>A.niger</i>)	<i>gpdA</i>	3000	Punt <i>et al.</i> , 1990
<i>A.nidulans</i>	Acid phosphatase (<i>A.niger</i>)	<i>pacA</i>	?	MacRae <i>et al.</i> , 1988
	Aspartyl protease (<i>M.miehei</i>)	all gene	?	Gray <i>et al.</i> , 1986
	Glucoamylase (<i>A.niger</i>)	<i>alcA</i>	?	Gwynne <i>et al.</i> , 1987
	(<i>A.niger</i>)	<i>gpdA</i>	5	Punt <i>et al.</i> , 1990
	Glucose oxidase (<i>A.niger</i>)	<i>god</i>	?	Whittington <i>et al.</i> , 1990
<i>A.niger</i>	Lactonase (<i>A.niger</i>)	<i>goxB</i>	?	Witteveen <i>et al.</i> , 1992
	Alpha-sarcin (<i>A.giganteus</i>)	<i>sar</i>	0.2	Wnendt <i>et al.</i> , 1993
	Lipase (<i>R.miehei</i>)	<i>amy</i>	?	Boel & Huge-Jensen, 1989
	Penicillin V (<i>P.chrysogenum</i>)	gene cluster	2.3	Smith <i>et al.</i> , 1990
<i>A.oryzae</i>	Aspartyl protease (<i>R.miehei</i>)	<i>amy</i>	3000	Christensen <i>et al.</i> , 1988
	Lipase (<i>R.miehei</i>)	<i>amy</i>	?	Boel <i>et al.</i> , 1987 Huge-Jensen <i>et al.</i> , 1989
<i>F.oxysporium</i>	Chitinase (<i>A.album</i>)	all gene	?	Blaiseau <i>et al.</i> , 1992
<i>M.circinelloides</i>	Aspartyl protease (<i>M.miehei</i>)	all gene	?	Dickinson <i>et al.</i> , 1987
<i>N.crassa</i>	Penicillin V (<i>P.chrysogenum</i>)	gene cluster	0.17	Smith <i>et al.</i> , 1990
<i>T.reesei</i>	Alpha amylase (<i>A.oryzae</i>)	<i>cbh1</i>	?	Cheng & Udaka, 1991
	Glucoamylase (<i>H.resinae</i>)	<i>cbh1</i>	710	Joutsjoki <i>et al.</i> , 1993a
	Invertase (<i>A.niger</i>)	<i>suc1</i>	?	Berges <i>et al.</i> , 1993

? Information not provided.

sole carbon source. Moreover, this transformant had two-fold and five-fold improvement in glucoamylase and RSD activities respectively, when it was cultivated in rice-koji (solid state culture). Strain improvement in *A.oryzae* has also been achieved by the reintroduction of its glucoamylase gene (Hata et al., 1991b). Indeed, homologous transformation has permitted several fungal proteins of commercial importance, such as *A.niger* glucoamylase (Verdoes et al., 1993), catalase (Fowler et al., 1993) and polygalacturonase (Bussink et al., 1991) and *Cryphonectria parasitica* aspartic protease (Choi et al., 1993) to be over-expressed by increasing the number of copies of the respective genes in host strains.

Another successful example of fungal heterologous expression is the expression of *R.miehei* aspartyl proteinase in *A.oryzae* under the control of α -amylase promoter that yields 3 g/l of slightly hyperglycosylated active protein in the medium (Christensen et al., 1988).

Heterologous expression in fungi might also provide an alternative way to express high levels of important fungal proteins impeded by physiological barriers in the natural strain. For example, the fungus *Hormoconis resinae* has growth difficulties and low protein secretion capacity. However, high levels of secreted *H.resinae* glucoamylase protein (GAMP) were obtained by expressing the *gamp* gene under the control

of the *cbh1* promoter in *T.reesei* (Joutsjoki et al., 1993a,b). Finally, there is a tendency to express fungal proteins in fungi that are genetically well characterized or industrially important such as *A. nidulans* and *A.niger* (MacRae et al., 1988; Smith et al., 1990; Wnendt et al., 1993; Blaiseau et al., 1992; Koch et al., 1993; Uusitalo et al., 1991).

3.3 Various other eukaryotic genes in fungi

Although filamentous fungi, such as *Aspergillus* species, have the capacity to secrete large amount of natural proteins, the yield achieved for non-fungal heterologous proteins has been very low (Table 1.8).

The one important determinant of the success in expressing heterologous genes is thought to be the promoter used to control the foreign gene. For example, *Aspergillus niger* transformed with the hen egg white lysozyme (HEWL) gene under the control of the *S.cerevisiae* alcohol dehydrogenase (ADH1) or phosphoglycerate kinase (PGK) promoters has produced no detectable lysozyme in the culture filtrates. In contrast, secreted lysozyme was found when the same gene was expressed under the control of the constitutive *A.nidulans* *gpdA* promoter (1 mg/l) or the inducible *A.awamori* *gam* promoter (12 mg/l) (Archer et al., 1990a).

Table 1.8 Non-fungal eukaryotic proteins produced in filamentous fungi

Host	Protein (Source)	Promoter	Yield/ l	References
<i>A.awamori</i>	Cathepin D	?	?	van den Hondel <i>et al.</i> , 1991
	Chymosin (calf)	<i>amyA</i>	70 mg	Korman <i>et al.</i> , 1990
		<i>glaA</i>	40 mg	Ward 1989b
		<i>glaA</i>	1 g	Dunn-Coleman <i>et al.</i> , 1991
	Thaumatococin (plant)	?	?	van den Hondel <i>et al.</i> , 1992
<i>A.nidulans</i>	a-1-anti-trypsin (human)	<i>alcA</i>	1 mg	Upshall <i>et al.</i> , 1987
		<i>adhA</i>	1 mg	Upshall <i>et al.</i> , 1987
	Cattle tick surface	<i>amdS</i>	1.8 mg	Turnbull <i>et al.</i> , 1990
	Chymosin (calf)	<i>glaA</i>	2.5 ug	Cullen <i>et al.</i> , 1987
	Corticosteroid binding globulin (human)	<i>alcA</i>	?	Devchand & Gwynne, 1991
	Epidermal growth factor (human)	<i>alcA</i>	?	Devchand & Gwynne, 1991
	Granulocyte colony stimulating factor (human)	<i>alcA</i>	1 mg	Upshall <i>et al.</i> , 1987
	Granulocyte colony stimulating factor (human)	<i>adhA</i>	1 mg	Upshall <i>et al.</i> , 1987
	Growth hormone (human)	<i>alcA</i>	?	Devchand & Gwynne, 1991
	Interferon a-2 (human)	<i>alcA</i>	1 mg	Gwynne <i>et al.</i> , 1987
		<i>glaA</i>	3 ug	Gwynne <i>et al.</i> , 1987
	Interleukin-6 (human)	<i>glaA</i>	2 mg	Contreras <i>et al.</i> , 1991
	Lactoferrin (human)	<i>alcA</i>	5 mg	Ward <i>et al.</i> , 1992b
	Pathogenic surface antigen (avian)	?	2 mg	van den Hondel <i>et al.</i> , 1991
	Parathyroid hormone (human)	?	2 mg	van den Hondel <i>et al.</i> , 1991
	Superoxide dismutase (human)	<i>alcA</i>	700 mg	van den Hondel <i>et al.</i> , 1991
	Tissue plasminogen activator (human)	<i>alcA</i>	1 mg	Upshall <i>et al.</i> , 1987
	Tissue plasminogen activator (human)	<i>adhA</i>	1 mg	Upshall <i>et al.</i> , 1987
	Tissue plasminogen activator (human)	<i>triA</i>	100 ug	Upshall <i>et al.</i> , 1987
<i>A.niger</i>	Chymosin (calf)	<i>glaA</i>	11 mg	van den Hondel <i>et al.</i> , 1992
	Egg white lysozyme (hen)	<i>gam</i>	12 mg	Archer <i>et al.</i> , 1990a
		<i>gpdA</i>	1 mg	Archer <i>et al.</i> , 1990a
	Interleukin-6 (human)	<i>gpdA</i>	15 mg	Broekhuijsen <i>et al.</i> , 1991
	Pancreatic glycoprotein A (porcine)	<i>glaA</i>	10 mg	Roberts <i>et al.</i> , 1992
<i>A.oryzae</i>	Chymosin (calf)	<i>amy</i>	10 mg	Christensen <i>et al.</i> , 1989
	Lactoferrin (human)	<i>amy</i>	25 mg	Ward <i>et al.</i> , 1992a
	Lysozyme (human)	<i>amy</i>	1.2 mg	Tsuchiya <i>et al.</i> , 1992
<i>T.reesei</i>	Chymosin (calf)	<i>cbh1</i>	40 mg	Harkki <i>et al.</i> , 1989
	Immunoglobulin (murine)			
	light chain	<i>cbh1</i>	0.2 mg	Nyyssonen <i>et al.</i> , 1993
	Fab fragment	<i>cbh1</i>	1 mg	Nyyssonen <i>et al.</i> , 1993
	CBHI-Fab fragment	<i>cbh1</i>	150 mg	Nyyssonen <i>et al.</i> , 1993

? Information not provided

In addition to a homologous promoter, an inducible promoter appears to be involved in high expression levels. An example is the expression of human lactoferrin (hLF) gene in *A.oryzae* under the control of the homologous *amyII* promoter. Despite the hLF anti-fungal properties, *A.oryzae* was found to secrete up to 25 mg/l of lactoferrin in inducing condition. The extent of glycosylation and glycoprotein linkage was similar to authentic lactoferrin and the recombinant protein had iron-binding capacity (Ward *et al.*, 1992a). The hLF cDNA sequence has also been placed under the control of the *A.nidulans* *alcA* promoter. However, an *A.nidulans* transformant containing the highest copies of integrated hLF cDNA (20 copies) when grown in induced conditions was found to produce only 5 mg/l of hLF. Approximately 30 % (2 mg/l) was secreted into the medium (Ward *et al.*, 1992b).

In general, transformants carrying multiple copies of the expression cassette show higher protein production. However, a transcription limitation has been suggested as result of a shortage of specific regulatory proteins (Verdoes *et al.*, 1993). Moreover, the integration sites has been shown to influence the level of chymosin expression (Ward, 1989a,b).

The levels of extracellular human interleukin-6 and calf prochymosin, both produced by *A.nidulans*, were

also found to vary among constructions which employed different types of secretion signals. The respective mammalian signal sequences were found to be functional in *A.nidulans*, however, the fungal *A.niger glaA* signal sequence was more efficient (Carrez et al., 1990; Cullen et al., 1987). Surprisingly, the addition of six amino acids residues from the *glaA* propeptide between the *glaA* signal peptide and chymosin cDNA sequence reduced drastically the chymosin production. This was suggested to be a result of mRNA secondary structure formation which might have interfered in translation process.

Improvement in heterologous expression has been achieved by expressing foreign protein fused to a fungal protein. For instance, the expression of the glucoamylase/interleukin-6 fusion protein in *A.nidulans* and *A.niger* (see Table 1.8). A further improvement has been obtained by using a protease minus mutant as host strain. The porcine pancreatic phospholipase A₂ (PLA₂) was shown to be rapidly degraded by both intracellular and extracellular *A.niger* proteases. *A.niger* transformed with an expression cassette containing the proPLA₂ cDNA sequence downstream of the *A.niger glaA* promoter region produced no detectable secreted PLA₂. However, an *A.niger* protease deficient mutant transformed with expression cassette harbouring the proPLA₂ cDNA sequence fused to the entire *glaA* gene was found to secrete up to

10 mg/l of PLA₂ in culture filtrates (Roberts et al., 1992). Strain improvement has been shown to be essential for high production of heterologous protein. The production of calf chymosin in *Aspergillus* is an example of how to improve secretion of a heterologous protein in filamentous fungi.

For the past few years or so the researchers at Genencor (San Francisco, USA) have been working on the expression of bovine chymosin in *Aspergillus*. Calf chymosin is the enzyme of choice for cheese manufacture because it has relatively specific enzymatic activity on the Phe-Met bond in k-casein in milk. Chymosin is synthesized as a preprochymosin which has the first 16 amino acids removed upon entry into the ER. The zymogen prochymosin is secreted into the medium and at low pH is activated by autocatalytic digestion which removes 42 amino acids at the N-terminus forming the mature chymosin. A bacterial recombinant chymosin was the first food ingredient made via recombinant DNA technology to obtain GRAS (Generally Regarded as Safe) status for human consumption (Flamm, 1991). The first attempt at generating a recombinant fungal bovine chymosin was to transform *A.nidulans* with four different expression cassettes containing (1) the *A.niger glaA* promoter and signal sequences (pGRG1); (2) the *A.niger glaA* promoter, signal sequence and propeptide sequences (pGRG2); (3)

the *A.niger glaA* promoter, signal sequence, propeptide and the first 11 amino acid residues from the mature glucoamylase sequence (pGRG4) and (4) the *A.niger glaA* promoter and chymosin signal sequence (pGRG3). All these constructions were linked to the prochymosin cDNA sequence and *A.niger glaA* terminator. High yields of chymosin expression were found either when chymosin was linked to the *glaA* signal sequence or to the 11 amino acids of the mature glucoamylase. However, pGRG2 which lacks the 11 amino acids at the N-terminus of glucoamylase showed the lowest amount of expression. The yield of chymosin expression was not correlated with the integrated gene copy number, but it seemed to be a result of the differences in chromosomal location of the integrated expression cassette. The secreted active chymosin reached a level of 2.5 mg/l in starch inducing medium (Cullen *et al.*, 1987). Later, the expression of chymosin in *A.awamori* using pGRG3, which contains the chymosin signal sequence, suggested that the expression cassette had been efficiently transcribed judging by the same level of chymosin and *glaA* mRNA. The maximum level of expression was found to be 15 mg/l for chymosin and 3 g/l for glucoamylase. There were several transformants in which 90 % of the total chymosin produced was found intracellular as chymosin or prochymosin, but not preprochymosin, which suggested that the precursor crossed the ER and possibly was further degraded by

native proteases inside the cell. Moreover, the secreted chymosin seemed to be digested by the native *A. awamori* aspartyl protease. In attempting to improve secretion efficiency an expression cassette was constructed by fusion of the full length *glaA* gene to prochymosin cDNA sequence (pGAMpR) (Ward, 1990). pGAMpR transformed strains produced 150 mg/l of chymosin with a low proportion of the total amount of chymosin retained inside the cells. Another three-fold improvement in chymosin production was achieved by transforming an *A. awamori* mutant deficient in its natural aspartyl protease and glucoamylase with pGAMpR (Berka et al., 1990). Nevertheless, the glucoamylase production from the construct *glaA*-chymosin was seven-fold less than production from only the *glaA* control expression cassette (Ward, 1989a). One transformant obtained by transformation of the *glaA*⁻, *pep*⁻ mutant with pGAMpR, which produced 250 mg/l of chymosin, was selected for further improvement strategies. The first strategy consisted of seven rounds of random mutagenesis under U.V. light and screening for higher chymosin producers by a semi automatic liquid culture screen. The highest chymosin production was 500 mg/l, which represented an improvement of two-fold. The next step was to submit the best producer to 2-deoxy-D-glucose resistance mutagenesis. This mutation is thought to be involved in part with cell permeability. The mutants which were able

to grow on medium containing this toxic sugar were later selected by high levels of chymosin secretion. The maximum amount of extracellular chymosin detected was 800 to 1000 mg/l. Finally, further transformation experiments were carried out in order to improve the chymosin production by additional copies of pGAMP_R, however, there was no significant improvement in chymosin levels observed (1345 mg/l) with the increased number of integrated expression cassette (Dunn-Coleman et al., 1991; Bodie et al., 1992).

More recently the feasibility of using *Trichoderma reesei* to express and secrete genetically engineered forms of antibodies was examined (Nyyssonen et al., 1992). The approach consisted of linking the light chain cDNA (pEN304), or the heavy Fd (V_H and C_H1) chain cDNA (pAJ202), or the CBHI-Fab fragments (pEN209) to an expression cassette containing the *T.reesei cbh1* promoter, signal sequence and transcription terminator. The CBHI-Fab fragments consist of a fusion of the *cbh1* sequences encoding the core-linker region to the heavy Fd chain cDNA sequence. Two transformation strategies were adopted. In the first, a *T.reesei* strain was transformed with pEN304 which contains, as the selectable marker, the *S.hindustanus ble* gene under the control of the *A.nidulans gpd* promoter and *trpC* terminator. Light chain-containing transformants were

then retransformed with either pEN209 or pAJ202 which have the *A.nidulans amdS* gene as the selective marker. The other strategy involved co-transformation of *T.reesei* with pEN304 and either pEN209 or pAJ202 and selection by phleomycin resistance. Both strategies resulted in transformants which produced antibodies in similar amounts. The best yields were achieved with the Fab and CBHI-Fab producers cultivated on cellulase inducing medium (see Table 1.8) (Nyyssonen et al., 1993).

IV MAMMALIAN AND VIRAL GENES USED IN THIS STUDY

4.1 Human interleukin-6 (hIL-6)

Interleukin-6 (IL-6) is a cytokine produced in lymphoid and nonlymphoid cells, including monocytes, macrophages, fibroblasts and endothelial cells. Its synthesis can be induced in response to a variety of stimuli, such as tissue injury, bacterial or viral infection, or to inflammatory lymphokines derived from activated macrophages, namely interleukin-1 (IL-1) and tumour necrosis factor- α (TNF) (Shimizu et al., 1990; Durum & Mealy, 1990).

Human interleukin-6 has a wide range of biological activities which are summarized in Table 1.9 (Hirano et

al., 1986, 1987, 1990; Kishimoto & Hirano, 1988; Brakenhoff et al., 1989, 1990; Castell et al., 1989; Garman et al., 1987; Morrone et al., 1988; Poupart et al., 1987; Miyaura et al., 1988; Hirano, 1991).

Table 1.9 Biological activities of Interleukin-6.

A- Induction of differentiation

1. B-cell differentiation or secretory-type Ig production
2. T-cell differentiation
3. Macrophage differentiation
4. Neuronal cell differentiation
5. Megakaryocyte maturation
6. Induction of acute phase protein synthesis
7. Induction of IL- 2 production and IL- 2 receptor expression in T-cells

B- Effects on growth

Positive

1. T-cell
2. Hematopoietic stem cell
3. Mesangial cell
4. EBV-transformed B-cell
5. Hybridoma/Plasmocytoma/Myeloma

Negative

1. Myeloid leukemic cell lines
 2. Breast carcinoma cell lines
-

See text for references.

Moreover, IL-6 has been suggested to have immuno-response augmenting activity both *in vivo* and *in vitro* and this may be of clinical use in the treatment of cancer and immunodeficiency (Asagoe et al., 1988). The potential use of hIL-6 in the treatment of decreased

platelet production due to various causes is based on the fact that daily injections of hIL-6 induce an increase of platelets in a murine system (Ishibashi et al., 1989).

The human IL-6 gene consists of five exons and four introns and has been mapped to chromosome 7 (Sehgal et al., 1987). The hIL-6 gene has one unique open reading frame encoding a 212 amino acids precursor protein. The N-terminal proline-rich hydrophobic peptide consists of 28 amino acids and is cleaved between Ala-Pro residues during the processing forming a mature protein of 184 amino acids, with molecular weight of approximately 21 kDa (Hirano et al., 1986). The 3'-untranslated region has 424 nucleotides in front of poly A tail signal sequence (AATAAA). The 5'flanking region has a purine at position -3 and C residues at positions -5 and -2 (Haegeman et al., 1986). Three regulatory regions have been identified in the promoter region which respond, amongst other factors, to IL-1 and TNF.

There are multiple forms of the IL-6 protein with molecular masses ranging between 20 and 30 kDa (27.5; 24; 23.3; 22.5 and 21.8 kDa). The predominant form of human IL-6 (hIL-6) has been shown to have an apparent molecular mass of 24 kDa (Gross et al., 1989; Gauldie et al., 1987). The primary structure of hIL-6 has two potential N - glycosylation sites (Haegeman et al.,

1986; Hirano *et al.*, 1986). However, immunoprecipitation of monocyte cultures treated with 1-deoxymannojirimycin, a blocking agent of complex-type oligosaccharides synthesis, and incubated with endoglucosaminidase H, revealed only one N-linked oligosaccharide chain. Moreover, only the 27.5 kDa form of hIL-6 is N-glycosylated. Analysis of O-linked carbohydrates on the hIL-6 molecule using sialidase and endo- α -N-acetylgalactosamidase showed that the molecular heterogeneity of human monocyte IL-6 is mainly due to differences in O-glycosylation patterns. O-glycosidically bound carbohydrates have a Gal(β 1-3)GalNAc core to which only sialic acid is bound (reviewed by Gross *et al.*, 1989).

Over-production of hIL-6 is related to rheumatoid arthritis, cardiac myxomas, cervical cancer and other conditions (Asagoe *et al.*, 1988; Hirano *et al.*, 1990). The measurement of hIL-6 levels in these diseases can be important in diagnosis, and their treatment by the use of inhibitors of hIL-6 would be an option. Thus, the therapeutic potential of hIL-6 makes it to be very important commercially. Before hIL-6 can be used therapeutically it has to be produced and purified in large amount and in a biologically active form. For this reason, Human interleukin-6 has been expressed in different host cells. An expression vector was

constructed containing the *E.coli trp* (tryptophan synthetase) promoter followed by a DNA fragment encoding fused human growth hormone (hGH)/ hIL-6. The hGH cDNA sequence was fused to the hIL-6 by a factor Xa recognition sequences (Asagoe *et al.*, 1988). 100 mg of hGH/hIL-6 fusion protein in which 58 % was the hIL-6 protein, was produced from one litre of cell culture. However, only 3 mg of biologically active hIL-6 was recovered from purification steps which followed intracellular extraction and enzymatic cleavage of the factor Xa site, representing a yield efficiency of 5% of total cellular protein. hIL-6 protein was also expressed in *E.coli*, under control of the *trp* promoter, as a fusion protein with 21 amino acids of human interleukin 2 (hIL-2) protein separated by a kallikrein recognition site (Tonouchi *et al.*, 1988). As a result, from one litre of *E. coli* culture, 45 mg of biologically active purified hIL-6 was prepared, which represented 11.6 % of total cellular protein. Another *E.coli* expression system for recombinant hIL-6 was developed by Yasueda and colleagues (Yasueda *et al.*, 1990). The expression cassette consisted of the *E.coli trp* promoter, dual Shine- Dalgarno sequences and an A-T rich segment inserted in front of the initiation codon of hIL-6 cDNA sequence, in order to avoid mRNA secondary structure. From one litre of culture, 600 mg of hIL-6 was produced, representing about 20 % of the total cellular protein

before purification. Recently, a system for transporting hIL-6 into the periplasmic space of *E.coli* has been reported (Barthelemy et al., 1993). This system has advantages in avoiding the formation of inclusion bodies, which improves the recovery of hIL-6. The regulatory signals of the expression cassette consisted of lipoprotein promoter, the IPTG inducible lactose operon promoter operator and outer membrane protein A ribosome binding site and signal peptide sequences. pOMP-IL6 contained the Ala of the *ompA* signal peptide connected to Pro of the hIL-6 gene. However, *E.coli* strains containing pOMP-IL6 showed slow growth and lysis a few minutes after induction with IPTG. These findings suggested that Pro as the N-terminal amino acid residue of the mature hIL-6 protein blocked processing by signal peptidase. However, the addition of an Ala or the substitution of the N-terminal Pro by Gly at the N-terminus of mature hIL-6 protein resulted in expression of unprocessed and processed forms of hIL-6. Furthermore, by manipulating the temperature it was possible to keep the insoluble precursor and soluble processed protein in a 50/50 ratio. From one litre of cultures 8 to 10 mg of purified, biologically active, hIL-6 was obtained.

In addition, yeast expression systems have been used to produce recombinant human IL-6 protein which is

already on the market (Boehringer Mannheim catalogue, 1992).

In an attempt to express hIL-6 in *A.nidulans*, expression cassettes containing the mature hIL6-encoding gene fused to the *A.niger glaA* promoter through different sequences were constructed. The following sequences were used: (1) hIL6 signal peptide, (2) *A.niger glaA* signal peptide or (3) *A.niger glaA* signal peptide and pro-peptide sequences (Carrez *et al.*, 1990). Improved yields were achieved with the construct containing the *A.niger glaA* promoter and signal sequence fused to the cDNA sequence of the mature hIL6. However, even this construct provided only 25 µg/l of secreted, biologically active, hIL6. Subsequently, the production of 5 mg/l of hIL-6 was achieved in *A.nidulans* strains transformed with an expression cassette containing the entire *A.niger glaA* gene under the control of the *A. nidulans gpdA* promoter fused to the hIL-6 cDNA sequence through a KEX2-motif encoding DNA sequence (Contreras *et al.*, 1991). Using a similar expression cassette, the hIL6 protein has also been expressed and secreted by *A.niger*. The maximum production level was obtained in an *A.niger* protease-deficient mutant, which produced 15 mg per litre of hIL6 protein in a mature, active form (Broekhuijsen *et al.*, 1991). Preliminary studies have suggested that the filamentous fungus *Gibberella*

fugikoroï may be a promising host for expressing hIL-6 at high levels (Peukert, unpublished).

4.2 Haemagglutinin-neuraminidase from Simian Virus 5

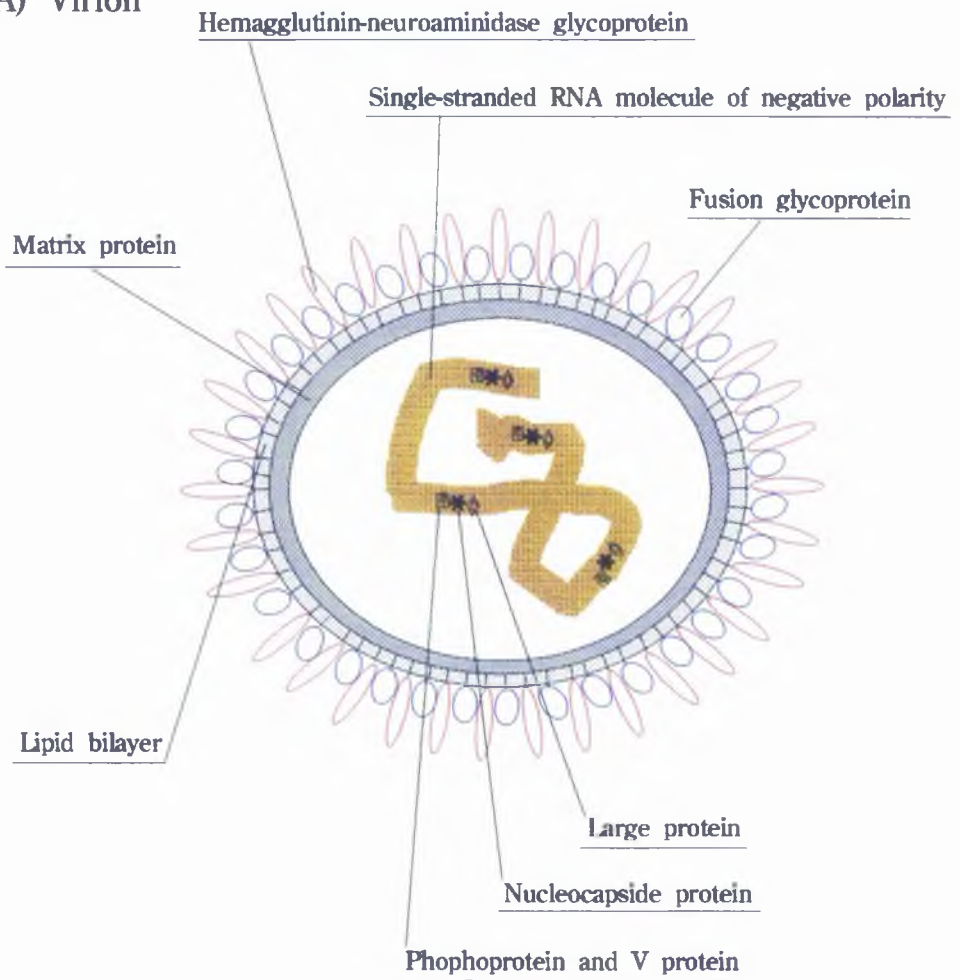
The Simian Virus 5 (SV5) is a prototype of the paramyxovirus family, closely related to the human parainfluenza virus types 2 and 4 and mumps virus (Precious et al., 1990). The viral genome consists of non-segmented single-strand RNA of negative polarity with approximately eight genes transcribed from approximately 15,000 nucleotides. There are six major structural proteins common to all members of the family. The genomic RNA is associated with the nucleocapsid protein (NP), phosphoprotein (P) and Large protein (L) forming the nucleocapsid structure. The nucleocapsid structure is package into an envelope during budding from the host cell. The envelope consists of a lipid bilayer derived from the host cell and through which protrude two viral glycoproteins, which form spike structures on the surface. A non-glycosylated viral protein, termed the matrix protein (M), is located on the inner membrane of the envelope. One of the glycoproteins is termed the fusion protein (F), which induces fusion of the viral particle to the cell, and this promotes virus entry. The other is the

haemagglutinin-neuraminidase (HN) glycoprotein which has receptor-binding, haemagglutinating and neuroaminidase activities. In addition, through its interaction with F protein, the HN protein also participates in the fusion process. The M protein is attached to the inner membrane through hydrophobic interactions and to the nucleocapsid through ionic interactions. The M protein directs the assembly of mature progeny during replication and regulates transcription by switching off transcription during assembly (Figure 1.3) (for a review see Galinski & Wechsler, 1991).

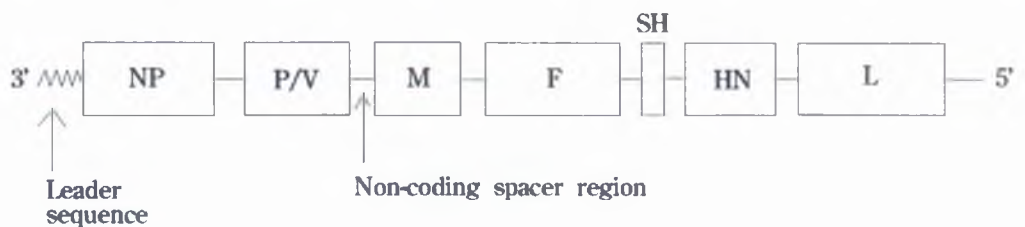
HN is classified as a type two glycoprotein. Hence it has an N-terminal cytoplasmic region and a C-terminal ectodomain. HN contains 565 amino acids comprising three major domains. The C-terminus hydrophilic domain is external to the membrane and contains carbohydrate molecules attached to it. Electron microscopy revealed that it has a globular head structure which is connected to a long slender stalk formed by 131 N-terminal amino acids (Morrison & Portner, 1991). All functional domains appear to be located on this globular domain. The second domain is 19 amino acids in length and is uncharged. This small domain consists of hydrophobic peptides which span the membrane. The third domain is also small, hydrophilic and is located inside the cytoplasm (Galinski & Wechsler, 1991 ; Morrison & Portner, 1991).

Figure 1.3 Schematic representation of the structural components and gene arrangement of Simian virus 5 (Young, 1987). See text for details.

A) Virion



B) Genome structure



The signal for type two orientation and retention of the N-terminus of HN protein in the cytoplasm has been proposed to be determined by the N-terminal positively charged residue flanking the signal/anchor (S/A) region (Parks & Lamb, 1991). One line of evidence supporting this fact was that the N-terminal domain of HN protein linked to S/A and C-terminal regions of the influenza virus M2 protein, a type III protein, induced type two orientation on the final M2 topology. Moreover, even though the N-terminal ectodomain of the M2 protein induced type III orientation to HN chimera at 60% efficiency, the type II orientation was restored by introducing positive charges into the N-terminal S/A flanking positions. The N-terminal positive charges within a signal sequence have also been proposed to be recognized by the *E. coli* SECA protein which is involved in protein translocation (Akita et al., 1990).

The folding of HN protein in mammalian cells has been shown to be completed 60 min after synthesis. The mature molecule is transported from the endoplasmic reticulum to the Golgi apparatus within 90 min after synthesis (Parks & Lamb, 1990a,b). Only folded HN molecules are transported through the exocytic pathway. Misfolded molecules associate stably with GRP78-Bip which retains the proteins in the ER until the molecules

can be properly refolded or be degraded (Ng *et al.*, 1990).

A secreted form of HN protein (HNF) has been obtained by replacing the HN S/A domain with a fusion related external domain (FRED) of the F1 subunit of the SV5 fusion protein which acts as a cleavable signal sequence. This HNF protein expressed in mammalian cells showed that the majority of secreted HNF were disulfide-linked dimers and enzymatically active whereas those molecules which were not dimers were degraded intracellularly (Parks & Lamb, 1990a). The slow dimerization of the soluble HNF molecules observed led to a hypothesis that if the HNF signal sequence was cleaved before folding and/or oligomerization, the molecules went into solution making oligomerization kinetically unfavorable. Furthermore, the transport of HNF from the endoplasmic reticulum to the Golgi apparatus has been shown to be the rate-limiting step in intracellular transport. Nevertheless, the attachment of complex carbohydrate chains to HNF dimers was not a prerequisite for secretion (Parks & Lamb, 1990a). In contrast, a secreted form of neuroaminidase (NAF) protein from influenza virus has also been obtained by replacing the natural N-terminal S/A domain of NA with FRED, but incompletely folded and nontetrameric forms of NAF were transported intracellularly and were secreted

into the medium. However, only the tetrameric form of NAF showed biological activity (Paterson et al., 1985). It has been suggested that although proper folding and oligomerization of membrane-bound proteins are required for intracellular transport the same does not occur for soluble forms and the transport kinetics depend on the protein under examination (Paterson & Lamb, 1990)..

There are six potential N-linked glycosylation sites on the HN ectodomain based on the amino acids consensus sequence Asn-X-Thr/Ser. However, two of them are not glycosylated probably due to a Pro residue at the X position of the consensus sequence. Deletion of single and multiple glycosylation sites on the HN protein showed that the glycosylation site at amino acid position 241 plays a key role in the HN folding. However, other glycosylation sites were also important in the process of HN folding and were probably involved in stabilizing the molecule (Ng et al., 1990).

V RESEARCH OBJECTIVES

The purpose of this work is to examine the feasibility of using *Aspergillus* species to produce large amounts of authentic foreign proteins.

First, the production of the human interleukin-6 protein by *A.oryzae* was assessed.

Second, an expression vector was constructed using the entire *A.oryzae amy3* gene and was subsequently used to express the Simian virus 5 Haemagglutinin-neuraminidase cDNA gene.

Third, the production of authentic viral glycoproteins by *A.oryzae* was investigated. For these studies the Haemagglutinin-neuraminidase protein from Simian virus 5 was used as a model.

CHAPTER 2

MATERIALS AND METHODS

2.1 Strains and Plasmid

2.1.1 Bacteria

Escherichia coli, strain DH5 α [F^- , *supE44*, *A(lacZYA - argF)U169*, ($\phi 80dlacZ \Delta M15$), *hsdR17*, *recA1*, *endA1*, *gyrA96*, *thi-1*, *relA1*, *deoR*], a recombination-deficient strain, was used for plasmid amplification and subcloning procedures. This strain contains the $\phi 80dlacZ/\Delta M15$ marker which allows screening for the disruption of the cloning vector β -galactosidase gene in recombinant colonies grown in the presence of X-gal and IPTG by observation of white / blue colonies. It carries *recA1* for insert stability of recombinant molecules and *endA1* for improving the quality of plasmid DNA. Finally, the strain is restriction defective (*hsdR17*) (Sambrook et al, 1989).

2.1.2 Filamentous fungi

Aspergillus oryzae IMI 144242 (ATCC = 91002), α -amylase over producing wild type. Strain *niaD14*, an nitrate reductase deficient strain (Unkles et al., 1989b) was used for *niaD* transformation. Strain 1560-6 (NOVO Industries, Denmark) an α -amylase over-producing and *argB* $^-$ mutant was provided by Dr C.A.Batt, Cornell

University New York was used for *argB* transformation. *A.oryzae* RIB40 wild type and strain $\Delta S537$, an *amdS*⁻ mutant which has its *amdS* gene disrupted, were obtained from Dr Gomi (Research Institute of Brewing Resources Co., Ltd., Japan). *Aspergillus sojae* RIB 1045 (ATCC=42251) which contains only one amylase encoded gene was also provided by Dr Gomi.

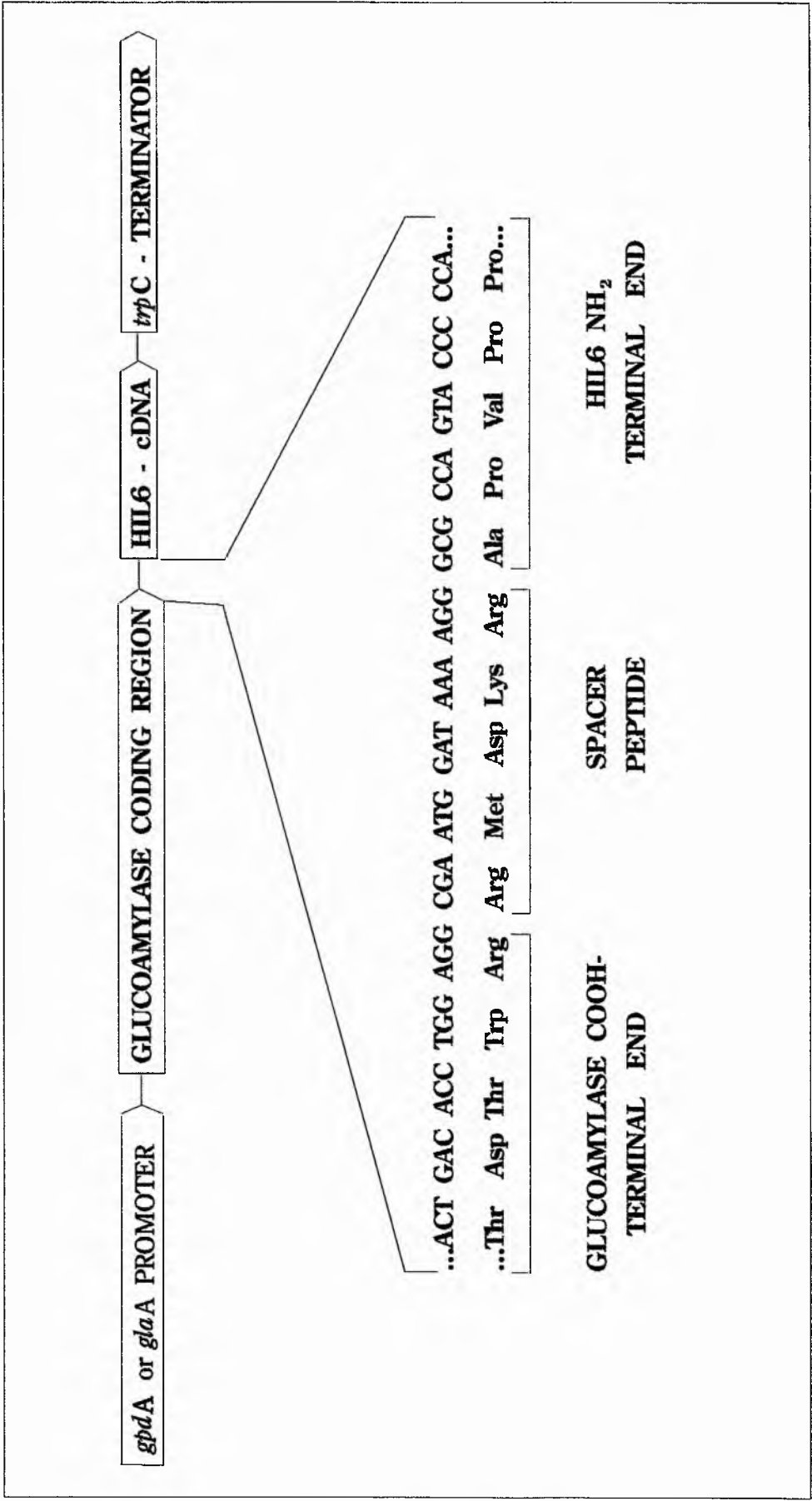
2.1.3 Plasmids

Table 2.1 Plasmids used in this study.

Vectors	Gene	References
pUC18		Sambrook, 1989
pAN56-30	<i>A.niger glaA</i>	Van den Hondel Pers. comm.
pSTA1210	<i>A.oryzae amy-3</i>	This work
p3SR2	<i>A.nidulans amdS</i>	Kelly and Hynes 1985
pSTA4	<i>A.nidulans argB</i>	Jonhstone et al. 1990
pSTA14	<i>A.oryzae niaD</i>	Unkles et al., 1989b
pFGLAhIL6T	<i>A.niger gla</i> + human IL-6 cDNA	Contreras et al. 1991
pFGPDGLAhIL6	<i>A.niger gla</i> + human IL-6 cDNA	Contreras et al. 1991
pAN56-3	<i>A.niger gla</i> + human IL-6 cDNA	Van den Hondel Pers. comm.
pAN56-4	<i>A.niger gla</i> + human IL-6 cDNA	Van den Hondel Pers. comm.
pSTA900	<i>A.oryzae amy3</i> gene	Macro, 1991
pGEXchNTAG	Simiam virus 5 haemagglutinin/ neuraminidase cDNA	Hanke et al., Pers. comm.

The expression vectors pFGLAhIL6T and pFGPDGLAhIL6 were generated by Fier's group at University of Ghent. These plasmid constructs are outlined in Figure 2.1.

Figure 2.1 Structure of pFGLAhIL6T and pFGPDGLAhIL6
expression cassettes (Contreras et al., 1991).



Both plasmids have the *A.niger glaA* coding region linked to the mature hIL-6 cDNA sequence. The intergenic DNA sequence encodes a KEX2-site, which enables hIL-6 to be released from the hybrid protein. The first vector contains glucoamylase gene under the control of its own inducible promoter while the second construct contains the strong constitutive *A.nidulans gpdA* (glyceraldehyde phosphate dehydrogenase) promoter. The *A.nidulans trpC* (tryptophan synthetase) terminator was employed in both constructions (Carrez *et al.*, 1990).

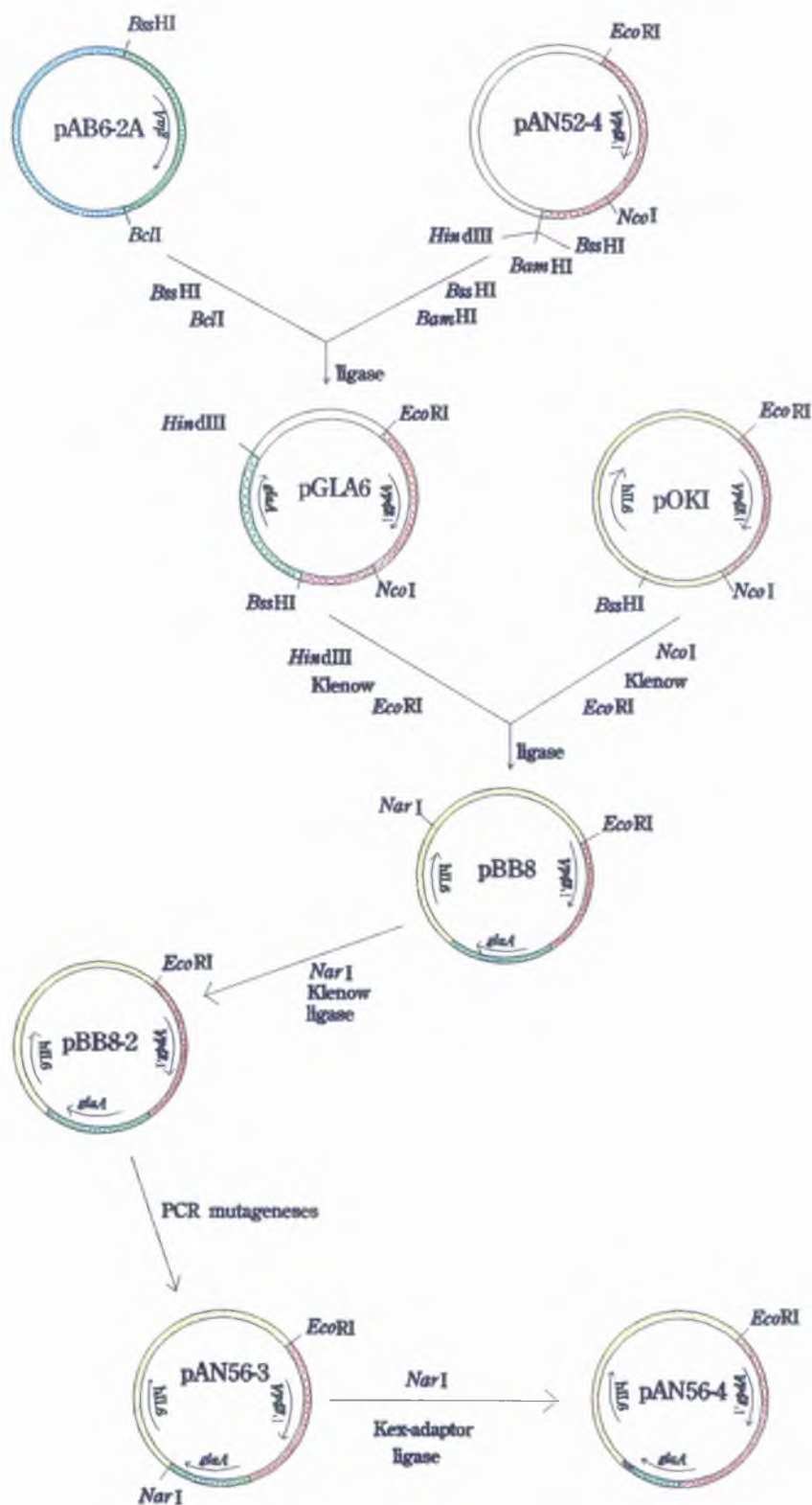
The vectors pAN56-3 and pAN56-4 were synthesized by van den Hondel's group at the TNO laboratory, Rijswijk (Figure 2.2). pAN56-3 harbours the mature hIL-6 cDNA sequence fused directly to the *A.niger glaA* coding region while pAN56-4 contains a intergenic sequence encoding a KEX2-site. The *glaA* gene are under the control of the *A.nidulans gpdA* promoter (C.A.M.J.J.van den Hondel, unpublished).

2.2 Reagents and Equipments

2.2.1 Reagents

The media constituents and solutions were purchased from Sigma or Lab M. The general purpose reagents and agar were acquisitions from BDH. 'Analar' grade reagents were obtained from BDH or Sigma.

Figure 2.2 Diagram of pAN56-3 and pAN56-4 construction
(van den Hondel, pers. commun.). Both pAN56-3 and pAN56-4
are approximately 8260 bp in size.



The digestion of the filamentous fungal cell-wall carried out in transformation procedures was promoted by Novozyme 234 supplied by Novo Industry, Denmark.

The restriction enzyme and DNA modifying enzymes were purchased by Northumbria Biological Ltd. plc, Boehringer Corporation Ltd or Pharmacia. SeaPlaque and Nuierve low melt agarose were provided by FMC Bio Products. DNA sequencing KIT was purchased by Sequi-Gen, Bio-Rad, Unit States Biochemical.

The anti-*A.niger* GLAA polyclonal antibody was graciously given to Dr J.R. Kinghorn by Dr C.A.M.J.J. van den Hondel and the anti-hIL6 polyclonal antibody by Dr R. Contreras.

Radioisotopes were acquired from ICN Biomedicals Inc..

2.2.2 Equipment

pH Meter: Pye Unicam pH Meter model 292MK2.

Shaker: The cultures were grown in an incubator shaker (New Brunswick Scientific Co. INC, model G25). The Rotatest Shaker (Luckham, model R100) was used for gently shaking gels in southern blot experiments and developing immunoreactions.

Incubator: Gallenkamp Economy Incubator size 1 was used at 24°C -37°C.

Centrifuges: Sorvall Instruments (DuPont), models RC5B or RC5C were employed in large scale plasmid extraction using Rotor GS-3 for harvesting the cells and Rotor SS-34 for pelleting DNA. In RNA extraction, Rotor HB-4 was used. Eppendorf Centrifuge 5415 was used in small scale centrifugation experiments. MSE clinical centrifuge with swing out rotor was used in protoplast preparation and in small scale fungal DNA extraction.

Spectrophotometer: Pye Unicam SP6 - 500 UV / VIS Spectrophotometer was used to measure cells concentration at 600 nm and nucleic acids at 260 and 280 nm wavelengths (Sambrook et al., 1989).

Lyophilization: Edwards Pirani 501 freeze dryer was employed for drying mycelia and nucleic acids.

Blotting/Immobilization: Nucleic acid immobilization was carried out on Hybond N Nylon membranes from Amersham International plc, UK. Proteins were immobilized on Nitrocellulose membrane (0.2 μ m) purchased from Schleicher and Schull, BA83, using the Multiphor II Nova Blot System, LKB 2117 - 005 / 250.

Electrophoresis: Horizontal gel rigs were from Bethesda Research Laboratories (BRL) and power was supplied from ATTA, AE3121 or ST1082 power pack models. Biorad vertical gel rig, PROTEAN II slab gel, was used in polyacrylamide gel electrophoresis of proteins. Biorad sequencing cell, model Sequi - Gen TM was used for sequencing gels.

Photographic materials: Photographs were taken with a Polaroid MP4 Land Camera using dark slides or Polaroid film.

Gel dryer: Bio-Rad Gel Dryer, model 583, was used for drying sequencing gels and polyacrylamide gels of proteins.

Film Processor: Fugi RX or Kodak XAR5 X - ray film was processed by a Fugi (RGII) X - ray film processor.

Polymerase Chain Reaction (PCR) machine: Hybaid Thermal Reactor model No.HB - TR1 was used in PCR protocols.

DNA synthesizer: DNA synthesizer, model 381A, Applied Biosystems, UK was used for synthesis of oligonucleotides.

2.3 Media

2.3.1 Bacterial media (Sambrook *et al.*, 1989)

2.3.1.1 Luria-Bertani Broth (LB)

NaCl	10	g
Tryptone	10	g
Yeast extract	5	g

Made up to 1 litre with dH₂O, adjusted to pH 7.5.

2.3.1.2 SOC medium

Glucose	20	mM
Tryptone	2	%
Yeast extract	0.5	%

NaCl	10	mM
KCl	2.5	mM
MgSO ₄	10	mM

Made up to 1 litre with dH₂O, adjusted to pH 7.5.

2.3.2 *Aspergillus* Media (Modified from Cove, 1966 and Clutterbuck, 1974).

2.3.2.1 Complete medium

Glucose	10	g
Peptone	2	g
Casein hydrolysate	1	g
Yeast extract	1	g
Salts solution	25	ml
Traces elements solution	1	ml
Vitamin solution	1	ml

Made up to 1 litre with dH₂O, adjusted to pH 6.5.

2.3.2.2 Minimal medium

Glucose	10	g
Salts solution	12.5	ml
Traces elements solution	1	ml

Made up to 1 litre with dH₂O, adjusted to pH 6.5.

A nitrogen source was added as required.

2.3.2.3 Salts solution

KCl	20.8	g
MgSO ₄ .7H ₂ O	20.8	g
KH ₂ PO ₄	60.8	g

Made up to 1 litre with dH₂O.

2.3.2.4 Trace elements (Hunter et al., 1950)

EDTA	50.0 g
ZnSO ₄ ·7H ₂ O	22.0 g
H ₃ BO ₄	11.1 g
FeSO ₄ ·7H ₂ O	5.0 g
MnCl ₂ ·4H ₂ O	5.0 g
CoCl ₂ ·6H ₂ O	1.6 g
SO ₄ ·5H ₂ O	1.6 g
Mo ₇ O ₂₄ ·4H ₂ O	1.1 g

Made up to 1 litre with dH₂O. Heated to 100°C stirred, cooled gradually to 60°C, then adjusted to pH 6.5-6.8 with KOH. Stored in light-proof bottle at 4°C.

2.3.2.5 Vitamin solution

Choline HCl	20.0 g
Biotin	2.5 g
Nicotinic acid	2.5 g
Riboflavin	2.5 g
Panthothenate Ca ⁺⁺ salt	2.0 g
Aneurine	1.5 g
Pyridoxine HCl	1.0 g
p-amino benzoic acid	0.8 g

Made up to 1 litre with dH₂O, sterilized by filtration.

2.3.2.6 Nitrogen source - stock solution

Acetamide	1	M
Adenine	1	M
Ammonium	1	M
Arginine	1	M
Glutamate	1	M
Hypoxanthine	2	mM
Nitrate	1	M
Nitrite	1	M
Semi skimmed Milk	10	%

For solid media 12 g of agar was added per litre. All the media and solutions were sterilized for 20 min, at 15 lb/sq.in. on liquid cycle except vitamin and amino acid solutions, which were filtered through 0.2 μ m ME of Syringe Tip Filter DynaGard supplied by Microgon Inc. Milk Solution was heated at 100°C for approximately 20 min, and left overnight at room temperature. The following day, the procedure was repeated.

2.4 General Techniques

2.4.1 Preparation of DEPC - water

RNA extraction requires a solution free from nucleic acids and proteins. Diethyl pyrocarbonate (DEPC) cross links proteins and also RNA, thus all solutions and water should be treated by DEPC, prior to use. A conical flask containing 5 l of water was treated with 0.4 % of DEPC, and stirred vigorously for about 3 h. All

the solutions were treated using DEPC-water and then autoclaved to destroy the vestiges of DEPC. As DEPC interacts with Tris-HCl, Tris-HCl containing solutions were treated with autoclaved DEPC-water.

2.4.2 Preparation of deionised formamide

Deionised formamide is required in the loading buffer of RNA extraction protocol. A volume of 100 ml of formamide was added to 25 g of 'Amberlite' monobed resin MB-1, analytical grade, and stirred for 2 h in a fume hood. After filtration through Whatman filter paper N°1, aliquots of 20 ml were stored at -20°C in light proof bottles.

2.4.3 Preparation of Tris saturated phenol

Phenol was melted at 60°C and an equal volume of 0.5 M Tris-HCl pH 8.0 and 0.1 % of hydroxyquinoline were added. Hydroxyquinoline is an antioxidant, a partial inhibitor of RNase and a weak chelator of metal ions (Sambrook et al., 1989). Moreover, hydroxyquinoline is yellow in colour, which facilitates the identification of the organic phase. The mixture was stirred for 30 min, then the upper phase was removed, and an equal volume of 0.1 M Tris-HCl pH 8.0 was added to the phenol. The mixture was stirred for 15 min, and then the upper phase was removed. The extraction was repeated until the

phenolic phase reached pH > 7.8. To the equilibrated phenol was added 0.1 volume of 0.1 M Tris-HCl pH 8.0 and 0.2 % of β -mercaptoethanol, and stored in dark bottles, at 4°C.

2.4.4 Siliconization of tubes and glass wool

Corex tubes and glass wool were washed in dimethyldiclorosilane solution, rinsed with an excess of tap water and DECON 90 detergent. Finally, siliconized glass wool was sterilized in 96 % ethanol and left to dry. Sterile siliconized glass wool was used in recovering DNA fragments from agarose. Siliconized tubes were baked for 8 h prior to use in RNA extractions.

2.5 General molecular biology techniques

2.5.1 Preparation of *Escherichia coli* competent cells

An Erlenmeyer flask containing 200 ml of Luria Broth medium was inoculated with 1 ml of an overnight culture of *DH5 α* cells, and incubated at 37°C with agitation. Cells were grown until the O.D. at 600 nm was approximately 0.25. The cells were harvested at 6,812 x g for 10 min at 4°C. The pellet was resuspended once in 100 ml of cold 100 mM MgCl₂ containing 5 mM Tris-HCl pH 7.4, and once in 100 ml of cold 100 mM CaCl₂ containing 5 mM Tris-HCl pH 7.4. The latter solution was

left to stand on ice for 20 min, and then, spun again. Finally, the pellet was resuspended in 2 ml of 100 mM CaCl_2 /14 % glycerol in 5 mM Tris-HCl pH 7.4. Aliquots of 200 μl were dispensed into pre-cooled 1.5 ml Eppendorf tubes and stored at -80°C .

2.5.2 Plasmid extraction - mini preparation

A single bacterial colony was inoculated in an Eppendorf tube containing 1 ml Luria Broth plus the required antibiotic and incubated with 300 RPM orbital shaking, at 37°C , overnight. The cells were harvested by low-speed centrifugation (3,180 x g for 1 min) and resuspended in 200 μl of lysis buffer (50 mM glucose; 25 mM Tris-HCl pH 8.0; 10 mM EDTA). After 5 min of incubation at room temperature, 400 μl of a freshly prepared alkaline solution (0.2 N NaOH; 1 % SDS) was added and mixed by inverting the tube. The tube was incubated on ice for 5 min. 300 μl of a 7.5 M ammonium acetate solution was added and incubation continued for a further 10 min to permit most of the protein, high molecular weight RNA and chromosomal DNA to precipitate. Centrifugation was carried out for 3 min at 15,000 x g. The clear supernatant was transferred to a tube and 0.6 volumes of isopropanol added. Plasmid was precipitated at room temperature for 30 min. Plasmid molecules were collected by centrifugation at 15,000 x g for 20 min. The pellet was washed with 70 % ethanol and left to dry

at room temperature. The dried pellet was resuspended in 50 μ l of TE (10 mM Tris pH 8; 1 mM EDTA) and 1 μ l of a 1 mg / ml solution of RNase was added. Incubation was carried out at 37°C for 1 h. When the DNA solution was turbid, it was centrifuged at 3,180 x g, for 2 min. However there was no need to discard the pellet, since it does not interfere with further manipulation of the DNA.

2.5.3 Plasmid Extraction - large preparation

An Erlenmeyer flask containing 100 ml of Luria Broth plus the required antibiotic was inoculated with 1 ml of an overnight culture and incubated at 37°C, overnight with agitation.

Cells were spun down at 6,812 x g for 10 min and resuspended in 4 ml of an isosmotic lysis solution (50 mM glucose; 25 mM Tris-HCl pH 8.0; 10 mM EDTA). After incubation at room temperature for 5 min, 8 ml of alkaline solution (0.2 N NaOH; 1 % SDS) were added and the tube placed on ice for 5 min. A cold alkali solution (6 ml of 3 M of KOAc pH 5.2) was added to disrupt base pairing and cause the linear chromosomal DNA of the host to denature and precipitate. Centrifugation was carried out at 17,210 x g, for 20 min, at 4°C. The supernatant, containing closed circular plasmid DNA molecules, which have their strands topologically intertwined and unable to be separated, was collected. The plasmid was

precipitated by adding 0.8 % of isopropanol and left at room temperature for 1 h. Plasmid molecules were harvested by centrifugation at $17,210 \times g$, for 20 min, at room temperature. The pellet was washed with 70 % ethanol and dried at room temperature. The DNA was resuspended in 4.5 ml of TE buffer and RNase added at a final concentration of $10 \mu\text{g/ml}$. Incubation was carried out at 37°C for 1 h.

2.5.4 Plasmid purification

Purified plasmid DNA may be obtained by different methods, such as equilibrium centrifugation in CsCl, ion exchange, gel filtration chromatography or differential precipitation using polyethylene glycol. Equilibrium centrifugation in CsCl is so far the most common methods used in the laboratories. It is based on differences in ethidium bromide-binding to linear and closed circular DNA molecules, causing different migration in CsCl gradients containing saturating amounts of ethidium bromide. Ethidium bromide intercalates between the base pairs, promoting the loss of the double helix spiral conformation, consequently increasing the length of the linear DNA. However the conformation of the closed circular plasmid DNA prevents the intercalation of additional molecules of ethidium bromide.

Purification of plasmid DNA was carried out using QIAGEN-pack 100. QIAGEN is an anion-exchange resin,

which is covalently linked to a silica gel base with a sophisticated hydrophilic surface coating to prevent nonspecific adsorption. The anion-exchange groups on the surface are carefully adjusted to give optimum separation in nucleic acid preparation (DIAGEN GmbH protocol, 1988).

The procedure requires three steps: (1) Adsorption of the lysate to QIAGEN. The column is equilibrated with buffer A (400 mM NaCl; 50 mM MOPS pH 7.0; 15 % ethanol; 0.15 % Triton X100), allowing adsorption of the DNA. (2) Washing away the impurities. The cartridge is washed with buffer C (1000 mM NaCl; 50 mM MOPS pH 7.0; 15 % ethanol) and (3) Elution of nucleic acid. The DNA is eluted with buffer F (1500 mM NaCl; 50 mM MOPS pH 7.5; 15 % ethanol). Finally, the DNA is precipitated and resuspended in 100 μ l of TE.

The plasmid concentration was determined by spectrophotometer, under U.V. light at O.D. of 260. The sample was prepared by taking 1 μ l of plasmid solution and adding it to 1000 μ l of water. The concentration was determined by the following formula:

$$[\text{DNA}] = \text{reading (260nm)} \times \text{dilution} \times 50 \mu\text{g} / \mu\text{l}$$

Purity of DNA preparation was estimated from the ratio obtained in O.D. 260 nm / O.D. 280 nm readings. The DNA is considered pure when the ratio ranges from 1.8 to 2.0.

2.5.5 Transformation of *E.coli* competent cells

Plasmid DNA (50 ng in a volume no greater than 10 μ l) was added to 200 μ l of *E.coli* competent cells, and mixed by gentle swirling. After 30 min incubation on ice, the tube was placed in a pre-heated bath at 42°C, for 90 sec followed by 2 min on ice. A volume of 800 μ l of SOC medium was added and the culture was incubated at 37°C for 1 h to allow the bacterial cells to recover and to express the antibiotic resistance marker encoded by the plasmid.

An aliquot of transformed competent cells was spread onto a selective medium (Luria broth - agar medium supplemented with the appropriate antibiotic) and left to dry at room temperature for a short period. The plates were incubated at 37°C, for about 16 h. When the β -galactosidase gene activity required to be observed, the selective medium was supplemented by X-gal (30 mg / 500 ml of medium dissolved in 1 ml of dimethylformamide) and IPTG (30 mg / 500 ml of medium dissolved in 1 ml of water).

2.5.6 Restriction endonuclease digestions

In the restriction enzyme reaction the salt concentration is important. The buffers supplied with the enzyme by the manufacturer were used whenever was possible. Double digestions were carried out

simultaneously when both enzymes have optimal activity in the same buffer. Table 2.2 shows most of the enzymes used in this study.

Table 2.2 Restriction endonucleases

Enzyme	5' Termini ^a	Buffer type ^b
<i>Bam</i> HI	protruding	L
<i>Bgl</i> II	protruding	L
<i>Eco</i> RI	protruding	L
<i>Eco</i> RV	blunt	H
<i>Hind</i> III	protruding	L
<i>Nhe</i> I	protruding	H
<i>Nru</i> I	blunt	H
<i>Sma</i> I	blunt	K
<i>Sph</i> I	protruding	L
<i>Stu</i> I	blunt	H
<i>Xba</i> I	protruding	L
<i>Xma</i> I	blunt	L

^a type of end created by respective enzyme

^b required buffers: L indicates Low ionic strength and H High ionic strength, K denotes KCl was used instead of NaCl.

Double enzyme digestions, requiring buffers with different ionic strength, were carried using first the buffer with lower ionic strength. After cleavage was achieved, NaCl and the other enzyme were added. In this case, the following 10 x buffers were used.

O - no salt

Tris - HCl pH 7.4	100	mM
MgCl ₂	100	mM
Dithiotreitol	10	mM
Bovine Serum Albumin	1	mg/ml

L - low salt

10 X buffer O

NaCl	0.5	M
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H - high salt
 10 x buffer O
 NaCl 1.0 M

K - Potassium buffer (*Sma*I)
 10 x buffer O, excepting Tris pH 8.0
 KCl 200 mM

2.5.7 Electrophoresis

Electrophoresis is a technique based on the difference of the migration rate of a particle when subjected to an electric potential difference. The DNA migration on an agarose gel depends on the molecular size of the DNA and the agarose concentration. Agarose acts as a molecular sieve, where shorter DNA runs faster. Thus, by increasing the concentration of agarose in gel, the best resolution of smaller fragments is achieved. Table 2.3 shows the relationship between agarose concentration and the range of size DNA molecules which is efficiently separated.

The conformation of the DNA particle influences the migration. Linear plasmid DNA, closed circular plasmid DNA and supercoiled closed DNA show different migration in a gel, presumably rigid conformation impedes the migration through the agarose. Applied current, base composition and temperature also alter the migration (Sambrook et al., 1989).

Table 2.3 - Relationship between percentage of agarose in gel and efficient separation of a range of linear DNA molecules (from Sambrook et al., 1989).

Agarose in gel (%)	Efficiency of separation(kb)
0.6	20 - 1
0.7	10 - 0.8
0.9	7 - 0.5
1.5	4 - 0.2
2.0	3 - 0.1

The most common electrophoresis buffers used here are TAE (0.04 M Tris - acetate; 0.002 M EDTA) and TBE (0.089 M Tris-Borate; 0.089 M Boric acid; 0.002 M EDTA). The TBE has greater ionic strength than TAE, being more sieving, thus, DNA molecules separate more slowly and with sharper resolution. Ethidium bromide was added to the gel at a final concentration of 0.5 μg / ml. The DNA - loading buffer used was 35 % sucrose; 10 mM EDTA; 0.25 % bromophenol blue or xylene cyanol.

2.5.8 Purification of DNA fragments

2.5.8.1 Siliconized glass wool

After electrophoresis was carried out as described above, the desired fragment was cleaved under U.V. light and placed into a cast which was filled with 1 % low melting point agarose without ethidium bromide. Another electrophoresis was run at low voltage. A slice containing the fragment of interest was placed onto sterile siliconized glass wool in an 0.5 ml Eppendorf tube, which had a hole made in the bottom with a needle

size 1.00 x 50 mm. A slice of agarose was squeezed against the glass wool and incubated at -70°C for 1 h. This 0.5 ml tube was assembled into a 1.5 ml Eppendorf tube and centrifuged at $3,180 \times g$ for 10 min. Consequently, the DNA migrates with the buffer through the glass wool and is collected in the outer Eppendorf tube while the agarose is retained by the glass wool. Phenol - chloroform extraction and ethanol precipitation were then carried out.

2.5.8.2 Phenol extraction

A section of low melting point agarose containing the band of interest was placed into a pre-weighed Eppendorf tube. Five vol of TE (20:1) pH 8.0 were added and incubated for 5 min, at 65°C . The re-melted agarose was allowed to cool and then equal volume of phenol added. This was mixed and spun down at $15,000 \times g$, for 1 min. Extraction was continued once with phenol - chloroform and once with chloroform. DNA was precipitated with 0.2 vol 10 M ammonium acetate and 2 vol ethanol.

2.5.8.3 Glass-milk matrix

Glass-milk matrix permits double-stranded DNA to be isolated from agarose gels or from cell lysates and also be separated from salts, phenol or radioactive

nucleotides. PrepaGene (Pharmacia) was used when handling small amounts of DNA as described by the manufacturer. The procedure was carried out in three steps. First, DNA - binding to the glass-milk matrix in the presence of 4 M sodium iodine. Second, washing away the contaminants with a solution containing 10 mM Tris - HCl pH 7.5; 10 mM NaCl; 1 mM EDTA in 70 % ethanol. Third, elution of the DNA from the matrix.

2.5.8.4 Electroelution

After electrophoresis of a digested plasmid in appropriate buffer and agarose concentration, the fragment of interest was visualized under U.V. light. The gel was cut just in front of that DNA band, in order to create a well. As the electrophoresis was continued, the fragment migrated into the well where it was collected.

2.5.9 Dephosphorylation

Dephosphorylation was carried out in order to prevent reannealing of linearized plasmid DNA in cloning experiments. Digested vector was submitted to phenol-chloroform extraction, ethanol precipitation and then resuspended in 10 mM Tris-HCl pH 8.3. The reaction was set up as follows:

DNA	48	μ l
10 x CIP buffer	5	μ l
CIP (calf intestinal phosphatase)	1	μ l
10 x CIP buffer consists of:		
Tris-HCl pH 8.3	100	mM
MgCl ₂	10	mM
ZnCl ₂	10	mM

Dephosphorylation of protruding 5' termini was performed by incubation at 37°C for 30 min, whilst for blunt ends or recessed 5' termini, the incubation was carried out at 37°C for 15 min and at 65°C for 15 min. 1 μ l of CIP was added and re-incubated as before. The reaction was stopped by adding EDTA pH 8.0 to 5 mM heated at 65°C for 1 h. Phenol-chloroform extraction and precipitation using sodium acetate at pH 7.0 to avoid EDTA precipitation, were performed. DNA was resuspended in TE (1:0.1) pH 7.5.

2.5.10 Phosphorylation of oligonucleotide

Phosphorylation procedure was applied to restoring the terminal phosphate at the 5' end of oligonucleotides. T4 DNA kinase catalyses the transfer of the terminal phosphate of ATP to 5' hydroxyl termini of polynucleotides, oligonucleotides and 3' mononucleotides. The reaction was prepared as following:

oligonucleotide	1.5	μ l
rATP	3.0	μ l
10 x buffer	3.0	μ l
T4 polynucleotide kinase (10-20U)	2.0	μ l

distilled water	20.5	μ l
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Incubation was carried out for 30 min, at 37°C.

10 x T4 polynucleotide kinase buffer		
Tris - HCl pH 7.6	500	mM
MgCl ₂	100	mM
Dithiothreitol	50	mM
Spermidine HCl	1	mM
EDTA pH 8.0	1	mM

2.5.11 Ligation

The formation of a phosphodiester bond between juxtaposed 5' phosphoryl and 3'- hydroxyl termini in duplex DNA can be restored with a DNA ligase. Single-stranded nicks in duplex DNA are repaired and both blunt-ended and cohesive-ended restriction fragments of duplex DNA are joined. Incompatible cohesive termini can also be ligated by bacteriophage T4 DNA ligase after partial filling of the recessed 3' termini using the Klenow fragment of *E.coli* DNA polymerase I.

2.5.11.1 Ligation reaction

The ligation of double-digested vector was set up in six Eppendorf tubes per experiment as shows table 2.4.

Table 2.4 - Ligation experiment

tube ^a	1	2	3	4	5	6
vector (ng)	10	10	10	100	100	10
insert (ng)	-	-	-	10	10	100
10x buffer (μ l)	1	1	1	1	1	1
water to (μ l)	10	10	10	10	10	10
ligase(1U/ μ l)	1	1	1	-	1	1

^a Tube 1, cell competency (undigest plasmid)
 Tube 2, efficiency of vector single digestion
 Tube 3, efficiency of vector double digestion
 Tube 4, efficiency of ligase
 Tubes 5 and 6, efficiency of ligation regarding the amount in ng of fragments used.

In order to optimize DNA ligation for efficient transformation, PEG 8000 was added at a final concentration of 5 % (w/v) (King & Blakesley, 1986).

2.5.11.2 In - gel ligation

It is no longer necessary to recover and purify DNA prior to cloning, digestion and labelling of DNA, as enzymatic processing of nucleic acids directly in remelted agarose can be performed. However, the efficiency and reproducibility of particular reactions vary, being attributed often to inhibitors in the agarose (FMC Bio Products application bulletin, 1991).

A low melting temperature agarose produced with high purity permits in-gel ligation-transformation with little or no inhibition. The digested plasmid was subjected to 0.8 % agarose gel electrophoresis. The desired fragment was cut out, placed into a cast and filled with 1 % of low melting point agarose.

Electrophoresis was carried out at low voltage. A gel slice containing the fragment of interest was put in a pre-weighed Eppendorf tube. Sterile distilled water was added to a final concentration of 1 ng / μ l of DNA, and the reaction incubated at 65°C for 10 min, for re-melting. The reaction was set up as follows:

10 mM Tris-HCl pH 7.5	18 μ l
10 x T4 DNA ligase buffer	5 μ l
Vector (molar ratio vector : insert 3:1)	
T4 DNA ligase	1 U
Mixed gently with pipette	
Remelted agarose (25 ng)	25 μ l

The ligation reaction was incubated at room temperature for 3-4 hours. The following 10 x T4 DNA ligase buffer was used:

Tris-HCl pH 7.5	200 mM
MgCl ₂	50 mM
Dithiothreitol	50 mM
ATP, pH 7.6	10 mM
nuclease-free BSA	500 μ g/ml

Bacterial transformation was carried out by heating the ligation reaction at 68°C for 5 min. 10 μ l of the re-melted reaction was added to 10 μ l of 10 mM Tris-HCl pH 7.5 at 40°C. An aliquot of 2 μ l was used to transform 200 μ l of *E.coli* competent cells.

2.5.12 Klenow reaction

Klenow, a large fragment of DNA polymerase I with 5' - 3' polymerase activity and 3' - 5' exonuclease activity, was used to "fill-in" the *Hind*III site of pSTA1200 (see Results Section 2.3). The reaction was set as follows:

pSTA1200 <i>Hind</i> III digested (total 1 μ g)	8 μ l
10 x Restriction buffer L	1 μ l
2 mM dNTP mix	1 μ l
Klenow fragment (5 U/ μ l)	0.5 μ l

The reaction was incubated at room temperature for 15 min, then heated at 70°C for 10 min to inactivate the enzyme. DNA was purified by phenol extraction and precipitated with ethanol. The blunt ends generated by the Klenow reaction were then ligated.

2.6 Polymerase Chain Reaction (PCR) (for a review, Innis et al., 1990).

The Polymerase Chain Reaction is an important advancement in synthetic DNA probes. The system was developed by Mullis at the Cetus Corporation and was first applied to the amplification of human β -globin DNA.

PCR is an in vitro method for the enzymatic synthesis of specific sequences of the target DNA using

a thermostable DNA polymerase of bacterium *Thermus aquaticus* (Taq) in the presence of two specific synthetic oligonucleotide primers, which bind to the flanking region of the target DNA. DNA replication is carried out through repeated cycles of melting heteroduplex DNA, hybridization of the primers to their complementary sequences onto opposite strands of the target DNA, and extension of the annealed primers by the polymerase across the region between the primers.

2.6.1 PCR components

i) **Primers** - The selection of efficient and specific primers even following strict guide-lines, remains empirical. Most primers are between 18 and 30 bases in length. Their composition is about 50 % of G + C content with a random base distribution. Stretches of polypurines and polypyrimidines at the 3' end of the primers promotes mispriming. Furthermore, primers which are complementary to each other especially in their 3' overlaps, promote primer hybridization and elongation. Therefore, primer-dimer artifacts are produced and the yield of the desired product is reduced.

The melting temperature strand (T_m) between 55°C and 80°C for each primer is desired. For long primers (> 25), the T_m is calculated by the following equation (Sambrook *et al.*, 1989):

$TmS = 81.5 - 16.6 (\log[Na]) - 41.2 (G+C \%) + (600/N)$, where N is the number of bases and [Na] is the molar concentration of Na^+ .

For short primers (< 25), the TmS can be calculated by the rule-of-thumb (Itakura et al., 1984), where 2°C are attributed for A and T and 4°C for G and C.

In a standard reaction, primers are used at the concentration of 0.1-0.5 μM . High primer concentration in the reaction promotes mispriming, accumulation of nonspecific product and formation of primer-dimers even if the primers lack complementarity at the 3' end.

ii) **Template** - Due to the high specificity of PCR a small amount of template can be used, which is about 10^5 to 10^6 target molecules. Moreover, isolation of DNA without extensive purification is still suitable for use in PCR.

iii) **Deoxynucleotides (dNTPs)** - dNTPs are used at the final concentration of 20 - 200 μM . Specificity and fidelity of PCR are increased by using lower dNTP concentration. Higher and / or no uniform concentration of dNTPs can produce misincorporation errors.

iv) **Enzyme** - Taq DNA polymerase of a thermophilic bacterial species *Thermus aquaticus* strain YT1 which grows at 70°C to 75°C has a molecular weight of 93,910 and high temperature optimum (Topt) for DNA synthesis.

Depending on the nature of the DNA template, it has an apparent T_{opt} of 75°C to 80°C with a specific activity approaching 150 nucleotides per second per enzyme molecule. Its half-life at 97.5°C, 95°C and 92.5°C are respectively 5, 40 and 130 min.

Without a 3' to 5' exonuclease activity Taq DNA polymerase is not able to proof-read. However the high fidelity achieved is suggested as being a consequence of K_m and V_{max} discrimination. Innis and colleagues referred the extension of a mismatched primers/template by Taq DNA polymerase as being less efficiently than a correct primers/template.

Taq DNA polymerase is used at the concentration between 1 and 2.5 units per 100 μ l reaction when other parameters are optimum. Higher enzyme concentration can induce nonspecific background products, such as primer-dimers even in absence of 3' end complementarity.

v) **Buffer** - The most common buffer used in PCR has been 10 to 50 mM of Tris-HCl pH 8.3-8.8. To facilitate primer annealing up to 50 mM KCl is added. Gelatin or bovine serum albumin at the concentration of 100 μ g / ml and nonionic detergents such as TWEEN-20 at the concentration of 0.05 to 0.1 % are included to help stabilize the enzyme. Magnesium ion concentration is critical. It affects primer annealing, strand dissociation temperatures of template and PCR product, product specificity, formation of primer-dimer artifacts

and enzyme activity and fidelity. The reaction mixture should contain 0.5 to 2.5 mM of magnesium over the total of dNTPs concentration.

In general the reaction can be set up as follows:

Stock solution	Final concentration
10 x buffer	1 x
1.25 mM dNTP mix	50 μ M
10 μ M Primer 1	0.2 μ M
10 μ M Primer 2	0.2 μ M
Template	0, 1, 10, 100 ng
Taq DNA polymerase 5 U / μ l	0.5 μ l
H ₂ O to	100 μ l

2.6.2 PCR conditions

The condition for amplifying DNA fragments by PCR has to be optimized in each case, considering the concentration, length and conformation of template and concentration, length and melting temperature (T_m) of the primers.

Denaturation step allows the strand-separation of template and newly PCR product. The strand-separation temperature (T_{ss}) and the time required should be optimized to avoid unnecessary loss of enzyme activity using high T_{ss} and/or long time. Incomplete separation reduces the product yield. An appropriate denaturation condition is 94°C for 1 min, 95°C for 30 sec or 97°C for 15 sec.

Primer annealing temperature and time are determined by the nature of primers (length,

concentration and melting temperature). Generally, annealing temperature of 5°C below the T_m of the primers is assumed. However, it is the first parameter to be changed for optimization. Lower temperature should be tried in case of no product be detected. Normally temperatures ranging from 55°C to 72°C give the best yield. Stringent annealing temperature is desired for increasing specificity.

Primer extension is influenced by the length and concentration of template and reaction temperature. When optimizing a PCR, different concentration of template should be carried out to establish the appropriated amount of template which gives highest yield. A rate of nucleotide incorporation at 72°C is estimates to vary from 35 to 100 nucleotides per sec, depending upon the buffer, pH, salt concentration, and the nature of DNA template. Therefore, up to 2 kb fragment can be produced in 1 min at 72°C. An suitable temperature is between 65°C and 72°C. Low extension temperature in presence of high dNTP concentration favours misextension of primers and extension of misincorporated nucleotides.

The cycle number depends upon starting concentration of the template. Few cycles give low product yield, while many cycles can favours nonspecific background products. For 3×10^5 target molecules is recommended between 25 to 30 cycles.

2.6.3 PCR Application

PCR represents a faster isolation method for a desired fragment of DNA with less time and effort required. For cloning purpose, PCR product should be amplified at maximum of specificity avoiding to clone non-specific product. The inclusion of restriction sites at the 5' end of primers permits ligation of complementary protruding termini, which gives best results. The prior restriction digestion of the PCR product can be carried out directed in a large volume, however it should be purified before ligated, since the PCR constituents inhibit the reaction. A fragment with multiple sites can also be cloned efficiently by PCR amplification using C_{75}dGTP instead of dGTP. C_{75}dGTP amplified DNA seems resistant to cutting, consequent only the restriction sites in the primer sequences are recognized by the respective restriction enzyme.

2.7 Filamentous fungi techniques

2.7.1 Fungal cell protoplasting procedure

2.7.1.1 Method 1

Spores from a five-day old culture were inoculated in 200 ml MM and incubated with 300 RPM orbital shaking, at 30°C , for 12-14 h. The cells were harvested in sterile gauze and washed first with sterile distilled water and then with solution A [0.8 M MgSO_4 in 10 mM

phosphate buffer pH 5.8. A volume of 5 ml of mycelium was put into a sterile universal tube, resuspended with 20 ml of solution A and 0.1 g of Novozyme 234 was added. This cell suspension was incubated in an orbital shaking at 250 RPM, at 30°C, for 45 min. The debris cells were spun down at 2,400 x g for 10 min. The supernatant was taken in small volume (5 ml) into 20 ml of solution B [1.2 M sorbitol, 50 mM CaCl₂, 10 mM Tris-HCl, pH 7.5]. The protoplasts were spun down at 2,400 x g for 5 min and washed with solution B three times. The pellet was resuspended in 1 ml of solution B. 10 x diluted protoplasts were accounted in Haemocytometer on four large squares i.e. 64 small squares. A small square area in the Haemocytometer is 0.04 mm x 0.1 mm. The concentration was determined by the formula:

$$\text{protoplasts} / 64 \times 4 \times \text{dilution} \times 10^6$$

2.7.1.2 Method 2

Spores from five day-old culture were harvested with saline-TWEEN 80 solution by scraping the plate surface and inoculated into 200 ml of MM supplemented with 1 M of commercial sugar. This spores suspension was incubated with 300 RPM orbital shaking at 30°C for 14 h. Mycelium was harvested in a sterile muslin cloth and washed with 500 ml of cold solution of 0.6 M MgSO₄. Mycelium was resuspended in 5 ml of cold osmotic medium (1.2 M MgSO₄; 10 mM sodium phosphate buffer pH 7.0) and

incubated into an 150 ml Erlenmeyer flask with 1.5 ml Novozyme solution (20 mg in 1.5 ml of osmotic medium) on ice for 5 min. To this was added 0.25 ml of BSA solution (12 mg BSA in 1.0 ml osmotic medium). Incubation was carried out in an orbital shaking at 200 RPM at 30°C for 60 to 90 min. Protoplasts were liberated from mycelial debris by vigorous shaking and placed on ice for few min. This suspension was placed into a cool sterile corex tube and an overlay of equal volume of cold trapping buffer (0.6 M Sorbitol; 100 mM Tris-HCl, pH 7.0) was added without mixing both phases. The balanced tubes were spun at 4,000 x g for 20 min at 4°C on a Sorvall RC-5 HB4 swing-out rotor. The protoplast band was located in the interface and was removed to a sterile corex tube using a Pasteur pipette. An equal volume of cooled STC buffer (1.2 M Sorbitol, 10 mM Tris-HCl, pH 7.5; 10 mM CaCl₂) was added to the protoplast suspension and spun at 8,000 x g for 5 min in the same rotor. Protoplasts were resuspended in 50 μ l. The transformation mixture consisted of:

Plasmid (10 μ g)	X μ l
2 x STC buffer	X μ l
1 x STC buffer	to 50 μ l
Protoplasts	50 μ l

The uptaken of DNA by protoplasts was facilitated by adding 25 μ l of 60 % PEG 6000 solution and incubating on ice for 30 min. Next 1 ml of 60 % PEG 6,000 solution was added and incubation continued at room temperature for 30 min. Finally, 5 ml of 1 x STC buffer were added

to the reaction, mixed gently and spun at 8,000 x g for 5 min. Protoplasts were resuspended in 1 x STC buffer and spreaded on to selective medium.

2.7.2 Fungal transformation

Transformation or co-transformation experiments were carried out by adding to an Eppendorf tube, 0.1 ml of protoplasts at a concentration of 10^8 /ml, 10 μ l of the selective plasmid and non-selective plasmid and 12.5 μ l of PEG solution [50 % PEG 4,000; 50 mM CaCl_2 ; 10 mM Tris- HCl, pH 7.5]. The incubation was carried out on ice for 30 min, followed by addition of 1 ml of PEG 4,000 solution. The transformed protoplasts were diluted with 2 ml of solution B [1.2 M sorbitol; 50 mM CaCl_2 ; 10 mM tris-HCl, pH 7.5]. This suspension was added to the selective medium at 40°C and poured over a layer of bottom agar. The plates were allowed to dry at room temperature. Incubation was carried out at 30°C, for about 5 days.

The viability of the protoplasts was measured by streaking the 10^{-5} protoplast dilution onto non-selective medium. The negative control was proved by pouring the 10^{-1} protoplast dilution onto selective medium.

2.7.3 DNA extraction - mini preparation

2.7.3.1 Method 1 (Leach *et al.*, 1988)

A conical flask containing 50 ml of complete medium was inoculated with 1 ml of spore suspension and incubated with a 300 RPM orbital shaking, at 30°C, for 16-20 h. Mycelium was harvested using sterile muslin, washed with distilled water, frozen in liquid nitrogen and lyophilized overnight. A small amount of dried mycelium was put into a 12 x 75 mm glass tube and broken with glass rod. A volume of 0.7 ml of LETS buffer [0.1 M LiCl; 10 mM EDTA; 10 mM Tris-HCl, pH 8.0; 0.5 % SDS] was added and then glass beads (0.45 mm) were loaded until the top of the liquid. The tube was covered with parafilm and vortexed for 2 min at top speed. 1 ml of phenol: chloroform: isoamyl alcohol (25:24:1) was added to each tube. After vortex at medium speed for 20 sec, the glass beads and cell debris were spun down at 2,400 x g in an MSE clinical centrifuge, for 5 min. An aliquot of 500 μ l of the aqueous phase was removed and placed in an Eppendorf tube. DNA was ethanol precipitated. Restriction enzyme digestions were carried out in a large volume.

2.7.3.2 Method 2 (Raeder & Broda, 1985)

Transformants were grown in minimal medium containing 1 % glucose and 10 mM ammonium for 48 h at an

appropriate temperature. Mycelia were washed in 20 mM EDTA pH 8.0, harvested by filtration under vacuum and freeze dried overnight. Dried mycelia were ground in Eppendorf tubes using a glass rod. The powder was resuspended in 500 μ l of extraction buffer (200 mM Tris pH 8.5; 250 mM NaCl; 25 mM EDTA and 0.5 % SDS) by vortexing at full speed. 350 μ l of phenol and 150 μ l of chloroform were added and briefly vortexed. The samples were centrifuged at full speed in an Eppendorf centrifuge for 1 h. The upper aqueous phase was removed immediately and incubated for 10 to 30 min with RNase solution (0.1 μ g / ml final concentration) at 37°C. Chloroform extraction was carried out once and DNA was precipitated by adding 0.54 volume of isopropanol. DNA was recovered by centrifugation at 15,000 x g for 30 min and washed with 70 % ethanol.

2.7.4 DNA extraction - large preparation

2.7.4.1 Method 1 (Garber & Yoder, 1983)

Lyophilized mycelium (0.8 g) was ground in liquid nitrogen. The powder was resuspended in 10 ml buffer [0.5 M sucrose; 25 mM Tris-HCl, pH 7.5; 50 mM EDTA; 100 μ g / μ l final concentration of RNase]. Sterile 30 % sarkosyl was added to a final concentration of 2 - 4 %, and incubated at 60°C for 1 h. Cell debris were spun down at 17,210 x g for 10 min. To the supernatant was added 200 μ g / ml of proteinase K and incubated at

37°C, for 4 h to overnight. Centrifugation was carried out at 17,210 x g, for 10 min, at 4°C. An equal volume of 30 % PEG 6,000 in 1.5 M NaCl was added to the supernatant followed by incubation at 4°C for 1 h to overnight. Centrifugation was carried out at 17,210 x g, for 10 min. The pellet was resuspended in 1/10 vol TE (50:10), pH 8.0. An equal volume of phenol was added, mixed gently and spun at 8,000 x g, for 5 min, at 25°C. DNA was precipitated with 2 vol 96 % ethanol and 1/10 vol 3 M sodium acetate pH 5.2.

2.7.4.2 Method 2 (Kolar *et al.*, 1988)

Lyophilized mycelium (0.8 g) was ground in liquid nitrogen and placed into a sterile 30 ml centrifuge tube. To the powder was added 10 ml of extraction buffer. The buffer was prepared by autoclaving 0.2 M Tris HCl pH 8.5; 0.25 M NaCl; 0.05 M EDTA then adding 48 mg / ml of PAS and 8 mg/ml of TNS fresh before used. An equal volume of phenol: chloroform: isoamyl alcohol (25:24:1) was added and mixed. Centrifugation was carried out at 8,000 x g for 5 min. The aqueous phase was removed to a clean tube while 3 ml of extraction buffer was added to phenolic phase. Centrifugation was carried out as before and the aqueous phases were combined. Phenol-chloroform extraction was repeated at least three times. Following this, the preparation was chloroform extracted to remove vestiges of phenol. DNA

was precipitated by 2 vol of 96 % ethanol and 1/10 vol of 3 M sodium acetate pH 5.2. DNA was recovered by centrifugation at $17,210 \times g$ for 40 min, at 4°C . The DNA was resuspended in 500 μl of TE (10:1) pH 8.0 and ethanol precipitation was repeated. DNA was spooled from the interface, avoiding the harvesting of broken DNA. The DNA was spun down in microcentrifuge, washed with 70 % ethanol and resuspended in 200 μl TE.

2.7.5 Dot Blot

Dot blot is a rapid and easy method to detect the presence of specific DNA or RNA in samples. Nevertheless it does not determine the size of the hybridizing molecules or the position of integrated DNA molecules. The hybridization signal is the sum of all sequences hybridizing to the probe under the conditions used. A quick screening of the transformants can be done by a single dot blot. However, a standard dilution of known amounts of DNA of the positive transformants applied next to each other in dots of uniform diameter can be used in quantitative analysis.

DNA samples were boiled for 5 min then chilled on ice. 1 vol of 20 x SSC was added and aliquots of 2 μl were spotted onto a hybond N membrane pre-wetted with 10 x SSC. This membrane was allowed to dry between the loadings, avoiding the spread of DNA molecules. A filter wetted in 0.25 M KCl solution was placed over the

membrane for 2 min, then taken out and left to dry. A filter wetted in denaturing solution was put on the membrane for 5 min, and removed to allow the filter to dry. A filter soaked in neutralizing solution was placed on the membrane for 2 min and then removed. The membrane was left to dry at room temperature, and then placed DNA-up side down on a transilluminator (312 nm) for 5 min in order to fix the DNA to the membrane by UV-crosslinking.

2.7.6 Southern Blot

A method of transferring fragments of DNA from agarose gels to cellulose nitrate filters was described by Southern (Southern, 1975). It consists of passing through a gel a solution of high salt concentration which carries with it the DNA to be trapped in the cellulose nitrate membrane. The fraction of DNA retained increases with the salt concentration. At concentrations above 10 x SSC the DNA is almost completely retained, whereas a small proportion is leached out into the solutions during denaturation and neutralization steps and only traces remain in the gel after transfer.

After conventional electrophoresis, the gel was placed in a tray containing 0.25 M HCl, on a rotatest shaker, for 15 min to depurinate the DNA. This increases the efficiency of transferring large DNA molecules. The gel was then treated in denaturation solution [1.5 M

NaCl; 0.5 M NaOH] for 30 min at room temperature following by neutralization solution [1.5 M NaCl; 0.5 M Tris - HCl pH 7.2; 0.001 M EDTA] also for 30 min. The blot was set up on a platform in a tray containing 20 x SSC solution. Three layers of 20 x SSC [3 M NaCl; 0.3 M Trisodium citrate] saturated Whatman 3MM filter paper larger than the gel size were placed on the platform forming a wick. Air bubbles were removed by sliding a glass rod over the paper to avoid them breaking the liquid contact and stopping the flux through the gel. The gel was placed on the wick. Hybond- N membrane saturated with 20 x SSC was placed on to the gel. The rest of the exposed wick was surrounded with cling film to avoid the flux of 20 x SSC by passing the gel and force the 20 x SSC through the gel. A sheet of 20 x SSC soaked 3MM paper was put onto the membrane and two more dry sheets over this. A stack of paper towels was placed onto the 3MM paper and a glass plate positioned on top. The stack was allowed to sit for 1 h and a 0.75 - 1 kg weight was placed over the glass plate. The minimum time required for complete transfer depends on the size of the fragments and probably also on the gel concentration.

2.7.7 DNA - DNA Hybridization

The nylon membrane was wetted in distilled water and incubated in prehybridization / hybridization solution for 5 h in water bath, at an appropriated

temperature, with shaking. The prehybridization / hybridization solution was prepared as shown in Table 2.4.

Table 2.4 - Prehybridization / hybridization solution.

stock conc.	final conc.	volume used (ml)
H ₂ O		33
20 x SSPE	5 x	25
30 % PEG	6 %	20
10 % Marvel (milk)	0.5 %	5
10 % SDS	1.0 %	10
5 % Na ₂ P ₄ O ₇	0.1 %	2
5 mg/ml Sperm DNA	0.25 mg/ml	5

The probe was added to the solution and incubation was allowed to continue overnight permitting hybridization. For homologous hybridization, where a stringent condition is desired, hybridization was carried at 65°C while in relaxed conditions 54°C was used instead. The washing step was carried out with 5 x, 1 x and 0.2 x SSC containing 0.1 % SDS and 0.1 % Na₂P₄O₇ at stringent conditions and with 5 x SSC at relaxed conditions.

Preparation of probe:

Probe was prepared by random hexanucleotide priming kits available. The reaction consists in using a random hexanucleotide sequence to prime DNA synthesis on denatured template DNA at numerous sites along its

length (Feinberg & Vogelstein, 1983). The multiprime DNA labelling reaction utilizes the "Klenow" fragment of DNA polymerase I from *E.coli* to catalyse a nick translation reaction. Lacking in 5'-3' exonuclease activity, the "Klenow" fragment promotes the sequential addition of nucleotide residues to the 3'-hydroxyl terminus of a nick without the elimination of nucleotide units from the nick's 5'-phosphoryl terminus.

A very small amount of input DNA (25-50 ng) is required for very high specific activity DNA probes to be produced with relatively small quantities of added labelled nucleotides (Amersham protocol, 1987). The percentage of added label incorporated into DNA is maximum in the first 2 h of incubation. The reaction was set up as following:

H ₂ O	30	μl
DNA [50 ng / μl]	1	μl
Reaction mix	10	μl
Bovine serum albumin (DNase-free)	2	μl
Enzyme ('Klenow') (2.5 U / μl)	2	μl
³² PdCTP (10 μC / μl)	5	μl

The mixture was incubated for 2 h at room temperature. The DNA was boiled for 2 min before being added to the reaction. The reaction mix was prepared as following:

Buffer O - 1.25 M Tris pH 8.0; 0.125 M MgCl₂
 Buffer B - 2 M Hepes pH 6.6

Buffer C - 50 units hexanucleotides in 556 μ l TE
dNTP's - 100 mM

Buffer A - 1000 μ l of buffer O

18 μ l of β -mercaptoethanol undiluted

5 μ l of each dATP, dTTP and dGTP

Reaction mix - 250 μ l buffer A

100 μ l buffer B

150 μ l buffer C

Removal of unincorporated label:

The unreacted triphosphates were removed by passing the mixture over a column of Sephadex G50 (purchased from Pharmacia) equilibrated with 10 mM Tris-HCl, pH 8.0 and 1 mM EDTA. This mixture was collected in aliquots. These fractions were monitored for radioactivity. The labelled DNA was boiled for 10 min, and added to hybridization solution.

2.8 Analysis of the transformants

2.8.1 Growth conditions

Transformants screened by dot blot were grown in minimal medium with either glucose or maltose as the sole carbon source. 100 ml of medium was inoculated with a spore suspension from a five old culture, and incubated with 300 RPM orbital shaking at 30°C for 48 h.

The mycelium was harvested by filtration through sterile muslin and subjected to a large scale DNA extraction. 1 ml of liquid culture was collected into an Eppendorf tube, frozen in liquid nitrogen and left overnight in the freeze-drier to concentrate proteins for further analysis by Polyacrylamide gel electrophoresis (PAGE) and Western blotting.

2.8.2 Polyacrylamide gel electrophoresis of proteins

Slab gels were prepared by polymerisation of acrylamide monomers into long chains crosslinked by N,N'-methylene bis-acrylamide initiated by the catalyses of free radicals from ammonium persulphate by TEMED (N,N,N',N'-tetramethylethylenediamine). The final acrylamide concentration in the resolving gel was 12.5 % (Hames, 1981).

The proteins were dissociated into their polypeptide subunits by boiling the protein mixture for 5 min in the presence of excess sodium dodecyl sulphate (SDS) and a thiol reagent to cleave disulphide bonds. The majority of polypeptides bind SDS in a constant weight ratio (1.4 g SDS to 1 g polypeptide). SDS-polypeptide complexes provide such a negative charge that the intrinsic charges of the polypeptides are insignificant. Thus, with the same charge densities, the complexes migrate according to polypeptide size allowing to be determined the molecular weight of sample

polypeptides by reference to the mobility of polypeptides of known molecular weight under the same electrophoretic conditions.

All PAGE experiments were carried out using a discontinuous buffer system. On the top of the slab, the gel (stacking gel) has large pores with pH 6.7, while on the bottom the gel (resolving gel) has small pores with pH 8.8. In the stacking gel, loaded proteins are concentrated as a result of a gradient zone created by the different mobilities of glycine and Tris ions. In the resolving gel, proteins are left behind glycine and Tris ions and are separated by size. The slab gels were prepared as detailed in Table 2.6.

Table 2.6 - Buffers used in gels of polyacrylamide.

	Resolving buffer	Stacking buffer
H ₂ O	11.9	5.6
Acryl/Bis-Acrylamide (30:08)	12.5	1.25
0.5 M Tris-HCl pH 6.8		2.5
3 M Tris-HCl pH 8.8	3.75	
SDS (10 %)	0.3	0.1
Ammonium Per-sulphate (15 %)	1.5	0.5

All values are in ml.

Prior to loading, 15 μ l of TEMED were added to the gel mix. The reservoir buffer was prepared with 6 g Tris-OH, 28.8 g glycine, 20 ml of 10 % SDS made to 2 l with distilled water and pH adjusted to 8.15 - 8.3. The sample was prepared by resuspending the protein powder in 50 μ l of distilled water and adding 50 μ l of loading buffer [1 ml glycerol; 0.2 ml 10 % SDS, 0.1 ml bromophenol blue; 0.2 ml β -mercaptoethanol; 0.5 ml

running buffer]. The electrophoresis was carried out overnight at 100 V.

Gels were stained with coomassie blue G250 (0.1%) in water: methanol: glacial acetic acid (5:5:2) and destained in 30 % methanol, 10 % glacial acetic acid.

2.8.3 Western blot

2.8.3.1 Method 1

The transfer of proteins from SDS polyacrylamide gels was according the instruction manual of Multiphor II Nova Blot (LKB) using the discontinuous buffer system. The immunological reaction was developed as described in the Amersham leaflet. To summarize, the filter was incubated for 60 min in TBST [10 mM Tris-HCl pH 8.0; 150 mM NaCl; 0.05 % Tween 20] containing 4 % skimmed milk, with gentle shaking. The filter was then incubated in the TBST solution containing 1 % milk with the first antibody [dilution 1:10000], overnight, at room temperature, with gentle shaking. The following day, the filter was washed with TBST buffer for 20 min, at least three times. Next, the filter was placed into AP buffer [100 mM Tris pH 9.5; 100 mM NaCl; 5 mM $MgCl_2$] containing 1 % marvel and alkaline phosphatase linked goat antirabbit IgG [dilution 1:1000] and incubated for 2 h. The filter was washed as mentioned before. Finally, the reaction was developed within 5 min by adding 80 ml

of AP buffer solution supplemented with 26.4 mg of Nitro blue tetrazolium in 580 μ l of 70 % dimethylformamide and 8.4 mg of 5-bromo-4-Chloro-3-Indolyl phosphate in 168 μ l of sterile distilled water. The excess of reagents were removed by washing the filter in 500 ml of distilled water.

2.8.3.2 Method 2

Proteins blotted onto nitrocellulose membrane were incubated in PBS buffer containing 10 % dried skimmed milk (w/v) and shaken gently at room temperature for 1 h or at 4°C overnight. This filter was briefly washed in PBS buffer supplemented with 5 % dried skimmed milk, and then incubated with the appropriate dilution of antibody in PBS buffer containing 5 % dried skimmed milk at room temperature for 1 h. Then it was washed thoroughly in several changes of PBS buffer following by a 5 min wash in PBS containing 5 % marvel. Finally, the filter was incubated with peroxidase linked protein A at room temperature for 1h. Next this filter was washed as mentioned before. The immunocomplex was detected by incubating the filter with ECL reagents, (Amersham International plc, UK) for 30 sec. Then the filter was covered with acetate sheets and exposed to X-ray films for 15 min. Detection of immobilized immunocomplexes is processed in three steps: (1) Protein A linked to horseradish peroxidase (HRP) binds to the Fc region of

the antibody, (2) HRP/hydrogen peroxide, in alkaline condition, catalyzes oxidation of luminol and (3) luminol reaches an excited state which decays to ground state by emitting light. This light is then detected by a short exposure to an autoradiography film (ECL western blotting protocols).

2.8.4 Immunoprecipitation

Fungal cultures were filtered and centrifuged. The culture supernatant was incubated with specific antibody at 4°C for 4 h, shaking or rolling slowly. *Staphylococcus* protein A (Staph A) was added at the ratio of 20 μ l of 10 % solution to each 1 μ l of antibody used. Incubation was allowed to continue for further 4 h. The immunoprecipitate was obtained by centrifuging at low speed for 5 min. No less than three washings were carried out using immunoprecipitation buffer [0.1 M NaCl; 1 mM EDTA; 10 mM Tris pH 7.6; 1 % NP-40 detergent; 1 % azide and protease inhibitors if required]. The final pellet was resuspended in 30 μ l of SDS-loading buffer and submitted to PAGE and western blot. The antibody - Staph A complex can be formed before being added to the culture supernatant. In this case, the complex was washed three times before being added.

2.9 Cultures storage

Bacterial strains and clones were stored in 1 ml Luria broth containing 15 % sterile glycerol, at - 80°C. In addition, plasmids were kept in TE buffer (10 mM Tris-HCl, 1 mM EDTA) pH 8.0, at - 20°C.

The fungal strains and transformants were kept for short term storage on minimal media slopes. For long term storage, spores from a week old plate were harvested with sterile skimmed milk, adsorbed onto sterile silica gel and stored at 4°C (Morris et al., 1982).

CHAPTER 3

RESULTS

The purpose of this work is to study the feasibility of using *Aspergillus* species to produce large amounts of authentic foreign proteins. First, the production of the human interleukin-6 protein by *Aspergillus* was assessed. Second, efficient expression vectors were constructed using the entire *A.oryzae amy3* gene. Third, the production of authentic viral glycoproteins by *A.oryzae* was examined.

SECTION I:

EXPRESSION OF HUMAN INTERLEUKIN-6 IN *Aspergillus*

hIL6-containing expression vectors were introduced into *Aspergillus* recipient strains by means of cotransformation. Three selection system were used: nitrate and arginine prototrophy and acetamide utilisation. The prototrophic transformants used in this Section and their features are listed in Table 3.1.1.

1.1 Genetic Transformation

1.1.1 The *niaD* selection system

The nitrate reductase gene (*niaD*) encodes the apoenzyme that converts nitrate to nitrite (Fig. 3.1.1).

Table 3.1.1 Criteria for designation of *Aspergillus* transformants harbouring the hIL6 DNA sequence. hIL6-transformants were named based on the recipient strain and the structure of expression cassette used, i.e. the promoter used to control the expression of the cassette encoding genes .

Recipient strains	Selection systems	Transformants (a)	Expression cassettes		
			vectors	promoters	genes (b)
<i>A.oryzae niaD200</i>	nitrate	TD200gla	pFGLAhIL6T	<i>glaA</i>	<i>glaA</i> -hIL6
<i>A.oryzae niaD14</i>	nitrate	TD14gla	pFGLAhIL6T	<i>glaA</i>	<i>glaA</i> -hIL6
<i>A.oryzae niaD14</i>	nitrate	TD14gpd	pFGPDGLAhIL6	<i>gpdA</i>	<i>glaA</i> -hIL6
<i>A.oryzae niaD14</i>	nitrate	TD14-3	pAN56-3	<i>gpdA</i>	<i>glaA</i> -hIL6 (c)
<i>A.oryzae niaD14</i>	nitrate	TD14-4	pAN56-4	<i>gpdA</i>	<i>glaA</i> -hIL6
<i>A.sojae niaD500</i>	nitrate	TD500gla	pFGLAhIL6T	<i>glaA</i>	<i>glaA</i> -hIL6
<i>A.sojae niaD500</i>	nitrate	TD500gpd	pFGPDGLAhIL6	<i>gpdA</i>	<i>glaA</i> -hIL6
<i>A.oryzae 1560-6</i>	arginine	T1560gla	pFGLAhIL6T	<i>glaA</i>	<i>glaA</i> -hIL6
<i>A.oryzae 1560-6</i>	arginine	T1560gpd	pFGPDGLAhIL6	<i>gpdA</i>	<i>glaA</i> -hIL6
<i>A.oryzae 1560-6</i>	acetamide	T1560-AMDgla	pFGLAhIL6T	<i>glaA</i>	<i>glaA</i> -hIL6
<i>A.oryzae 1560-6</i>	acetamide	T1560-AMDgpd	pFGPDGLAhIL6	<i>gpdA</i>	<i>glaA</i> -hIL6

(a) Transformant number follows this general denomination.

(b) All expression cassettes contain the *A.niger* glucoamylase (*glaA*) gene fused to hIL-6 cDNA by an intergenic sequence encoding a KEX2-site.

(c) The intergenic sequence do not encode a KEX2-site..

Figure 3.1.1 The biochemistry and genetics of the conversion of nitrate to ammonium ions. Extracellular nitrate is transported to the cell by a transporter encoded by the *crnA* gene, converted to nitrite by the action of nitrate reductase encoded by *nirA* gene, and finally converted to ammonium ions by nitrite reductase, the *nirB* gene product. The nitrate assimilation is induced by the product of the *nirA* gene and repressed by the product of the *areA* gene. The product of the *cnxJ* gene controls the expression of the *cnxA-G* genes, which are involved in the synthesis of molybdenum cofactor (Cove, 1979; Unkles *et al.*, 1989a and 1991; Kinghorn, 1989; Scazzocchio & Arst, 1989).

EXTRACELLULAR

NITRATE $\xrightarrow[\text{Transporter}]{\text{cnxA}}$

identified control genes for
nialD expression

+ *nirA*
- *areA*
cnxJ

INTRACELLULAR

NITRATE $\xrightarrow[\text{Nitrate reductase}]{\text{nialD}}$ NITRITE $\xrightarrow[\text{Nitrite reductase}]{\text{nirA}}$ AMMONIUM

+

Molybdenum
cofactor
cnxA → G

This system offers many inherent advantages as the nitrate assimilation pathway is entirely dispensable and mutation in it should not alter growth or metabolic fluxes through biosynthetic pathways. Mutants may be easily obtained by positive selection on the basis of chlorate resistance, which can be done by spontaneous means, reducing the possibility of secondary mutations arising in genes of commercial interest or in genes encoding essential catalytic steps. Moreover, mutants in putative nitrate assimilation genes are screened by growth tests using different sources of nitrogen (Unkles et al., 1989a).

The *niaD* gene transfer system has been employed in the transformation of a number of filamentous fungi including *A. niger* (Campbell et al., 1989); *A. oryzae* (Unkles et al., 1989a,b); *Fusarium oxysporum* (Malardier et al., 1989); *Penicillium chrysogenum* (Whitehead et al., 1989); *Cephalosporium acremonium* (Whitehead et al., 1990); *Gibberella fugikuroi* (Sanchez-Fernandez et al., 1991) and several plant pathogens (Daboussi et al., 1989).

1.1.1.1 Isolation of *niaD* mutants

At least 10 different defective mutants in nitrate assimilation can be obtained by chlorate resistance: two are structural genes, the one is a permease for nitrate uptake (*crnA* gene) and the other is the structural gene

of nitrate reductase (*niaD* gene); the synthesis of the molybdenum co-factor required for nitrate reductase activity as well hypoxanthine dehydrogenase involves six loci (*cnxA* to *cnxJ* genes); and finally, the genes *areA* and *nirA*, which are required for the control of *niaD* expression. The *areA* and the *nirA* genes are involved in the repression and induction, respectively, of the nitrate assimilation system. The mechanism in which the chlorate resistance generates one specific genotype is as yet unclear. It seems that the level of toxicity of chlorate depends upon the sole nitrogen source employed in the selective medium (Cove, 1976). Furthermore, the nitrogen source used influences the type of mutation obtained, for example, *nirA* mutation was reported to be infrequent when glutamate is the nitrogen source used (Tomsett & Cove, 1979).

The toxic effects of chlorate on *A.oryzae* strain RIB40 and *A. sojae* strain RIB1045 were determined by inoculating both strains onto MM containing 10 mM of glutamate as sole nitrogen source supplemented with 100, 200 or 470 mM of chlorate. The relationship between dose and toxic effect was observed, with lower growth occurring on medium with higher chlorate concentration. Furthermore, strain RIB1045 showed higher sensitivity to chlorate than RIB40.

10^9 conidial spores from five day-old culture were inoculated onto agar MM containing 470 mM chlorate and

10 mM glutamate as the sole nitrogen source. The plates were incubated at 30°C for 10 days.

Sixty-six resistant colonies of *A.oryzae* RIB40 were obtained at different stage of growth as judged by colony size and sporulation. In random manner, 18 sporulating colonies were subcultured onto chlorate MM for purification. While all twenty-one resistant *A.sojae* RIB1045 colonies obtained were also purified. The nitrate reductase deficient mutants were screened on MM supplemented with different sources of sole nitrogen, namely glutamate, ammonium, nitrate or nitrite at a concentration of 10 mM and hypoxanthine or adenine at a concentration of 1 mM (Table 3.1.2). Results are shown in Table 3.1.3.

Table 3.1.2 Growth test for putative identification of mutation on chlorate resistant mutant.

Gene mutation ^a	<i>niaD</i> ⁻	<i>crnA</i> ⁻	<i>niiA</i> ⁻	<i>cnxA-J</i> ⁻	<i>areA</i> ⁻	<i>nirA</i> ⁻
Chlorate status	R	R	S	R	R	R
Growth on						
Nitrate	-	+	-	-	-	-
Nitrite	+	+	-	-	-	-
Ammonium	+	+	+	+	+	+
Hypoxanthine	+	+	+	-	-	+
Proline	+	+	+	+	-	+
Glutamate	+	+	+	+	-	+

- The minus superscripts denote loss of gene function

R Denotes resistance to chlorate

S Denotes sensitivity to chlorate

+

- Denotes poor growth

^a Gene mutation obtained by resistance to chlorate: *niaD*, nitrate reductase encoding gene; *crnA*, encoding nitrate permease gene; *niiA*, nitrite reductase encoding gene; *cnxA-J*, genes involved in molybdenum co-factor synthesis; *areA*, a negative control gene for nitrate assimilation and *nirA*, a positive control gene for nitrate assimilation.

Table 3.1.3 Frequency of putative genotypes of chlorate resistant mutants. The frequency (%) of different spontaneous putative mutants of *A.oryzae* RIB40 and *A.sojae* RIB1045 selected by resistance to 470 mM chlorate on glutamate minimal medium.

Strains	N° mutants selected	Genotypes identified		
		<i>niaD</i>	<i>crnA</i>	<i>nirA</i>
<i>A.oryzae</i> RIB40	18	2 (11)	2 (11)	14 (78)
<i>A.sojae</i> RIB1045	21	1 (5)	3 (14)	17 (81)

Frequency of putative mutants is shown in brackets

Two mutants derived from *A.oryzae* strain RIB40 designated *niaD200* and *niaD300*, and one mutant from *A.sojae* strain RIB1045, designated *niaD400* were tested for reversion. These chlorate resistant mutants were inoculated onto agar CM and incubated at 30°C for 5 days. 10^6 of viable spores were harvested and streaked onto nitrate MM. Incubation was carried out as before and no reversion of mutation was observed.

Despite the successful isolation of putative *niaD* mutants, there were neither *cnxA*⁻ mutants nor *areA*⁻ mutants generated by chlorate resistance. Furthermore, the putative *nirA* mutants in *A.oryzae* RIB40 and *A.sojae* RIB1045 represented about 80 % of the total mutants when glutamate was used as the sole nitrogen source in the selective medium.

In order to study the predominance of a genotype generated by chlorate resistance, *A.sojae* RIB1045 was submitted to another spontaneous mutagenesis using proline as the sole nitrogen source. The concentration

of chlorate and conditions were as described before. Growth tests of 20 chlorate resistant mutants showed an increased frequency of the *niaD* mutation. However, *nirA* mutation was still predominate (Table 3.1.4). This suggested that, beside the fact that the nitrogen source employed influences the type of putative mutation, the strain used may also exert some influences.

Table 3.1.4 Frequency of genotypes of chlorate resistant mutants. The frequency (%) of spontaneous putative mutants of *A.sojae* RIB1045 selected by resistance to 470 mM chlorate on proline minimal medium.

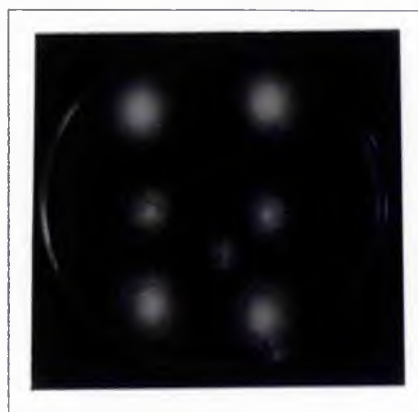
Strain	N°of mutants selected	Genotypes identified		
		<i>niaD</i>	<i>crnA</i>	<i>nirA</i>
<i>A.sojae</i> RIB1045	20	7 (35)	1 (5)	12 (60)

Frequency of putative mutants is shown in brackets.

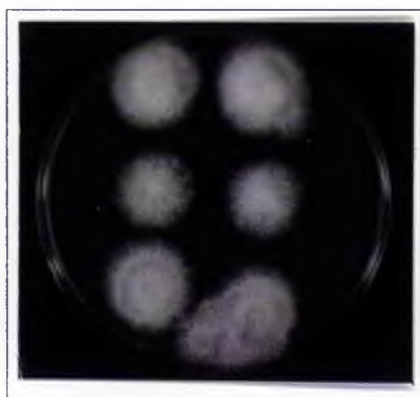
The *A.sojae* RIB1045 putative *niaD* gene mutants designated *niaD500*, *niaD600*, *niaD700*, *niaD800* and *niaD900* were tested for reversion. 10^6 viable spores from each mutants grown on CM were harvested and spread onto nitrate MM and incubated at 30°C for one week. Lack of reversion in all cases confirmed that the mutations were stable. Growth of *A.oryzae* RIB40 and *A.sojae* RIB1045 *niaD* mutants on agar MM supplemented with different nitrogen sources are shown in Figures 3.1.2 and 3.1.3, respectively. Mutants *niaD200* and *niaD500* were used in transformation experiments.

Transformation of the nitrate reductase deficient mutants *niaD200* (*A.oryzae* RIB40); *niaD14* (*A.oryzae*

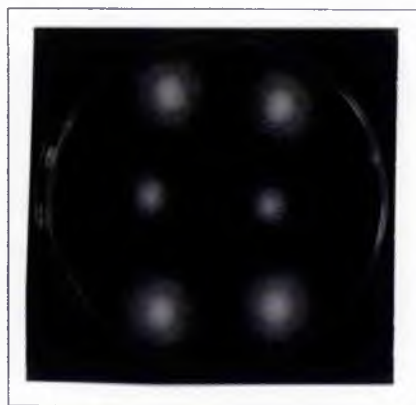
Figure 3.1.2 Chlorate resistant mutants of *A. oryzae* strain RIB40 grown onto MM containing different nitrogen sources.



ADENINE



AMMONIUM



HYPOXANTHINE

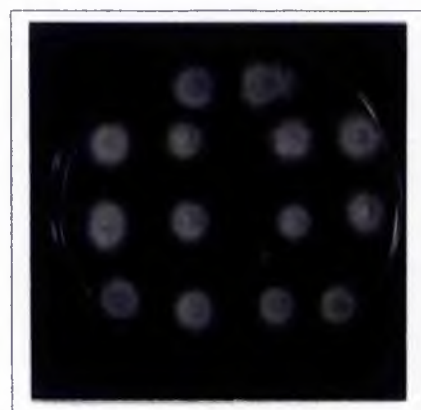
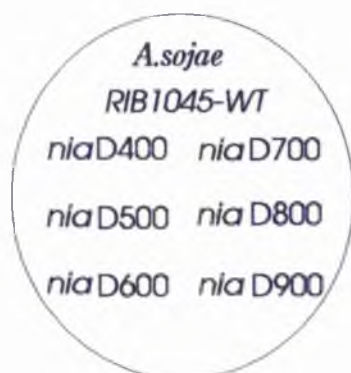


NITRITE



NITRATE

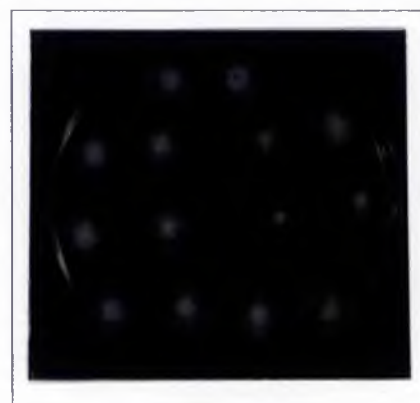
Figure 3.1.3 Chlorate resistant mutants of *A. sojae* strain RIB1045 grown onto MM containing different nitrogen sources.



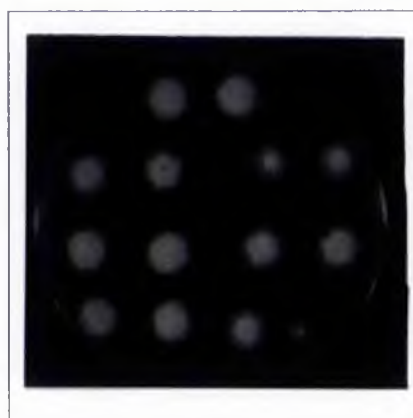
GLUTAMATE



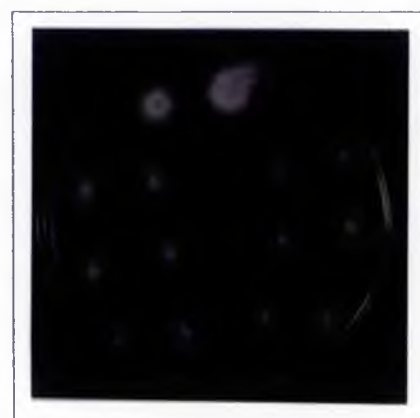
AMMONIUM



HYPOXANTHINE



NITRITE



NITRATE

STAN); and *niaD500* (*A. sojae* RIB1045) were carried out by complementation using the *A. oryzae niaD* gene cloned into pSTA14 (Unkles et al., 1989b).

1.1.1.2 Co-transformation using pFGLAhIL6T or pFGLAGPDhIL6

Protoplasts were prepared as described in Materials and Methods Section 2.7.1.1. Approximately 10^8 viable protoplasts were co-transformed with pFGLAhIL6T or pFGLAGPDhIL6. Approximately 6 μ g of selective plasmid and the same molar concentration of the non-selective plasmids were used to transform *A. oryzae niaD14* and *niaD200* strains. The molar concentration of non-selective plasmids used to transform *niaD500* strain was 1.5-fold higher than the selective one. The protoplasts were spread onto selective MM (minimal medium containing 10 mM nitrate as the sole nitrogen source and 1 M sorbitol as stabilizer). Transformants were grown in liquid MM. DNA mini-preparation was carried out on young mycelial cells. Approximately 1 μ g of genomic DNA was dot blotted onto nylon membranes and hybridized with the *EcoRI* / *HindIII* 1 kb fragment of pSP64GcHIL6 which contains part of the hIL-6 gene. The results are shown in Table 3.1.5. The transformation frequency efficiency represents the number of transformants obtained per 1 μ g of selective plasmid DNA used.

Table 3.1.5 Transformation frequencies of various *Aspergillus niaD* deficient mutants using *niaD* selection system.

Recipient ^a strains	Plasmids ^b used	Number ^c transf.	Transf. ^d frequency	Cotransf. ^e frequency
<i>niaD200</i>	pSTA14 +	4	0.6	NA
	pFGLAhIL6T	9	1.5	11 (1)
	pFGPDGLAhIL6	17	2.8	0 (0)
<i>niaD14</i>	pSTA14 +	4	0.6	NA
	pFGLAhIL6T	6	1.0	17 (1)
	pFGPDGLAhIL6	18	3.0	22 (4)
<i>niaD500</i>	pSTA14 +	6	1.0	NA
	pFGLAhIL6T	30	5.0	17 (5)
	pFGPDGLAhIL6	21	3.3	33 (7)

NA Not applicable

^a Chlorate resistant mutants of *A.oryzae* RIB40, *niaD200*; of *A.oryzae* STAN14, *niaD14* and of *A.sojae*, *niaD500*.

^b Selective and non-selective plasmids used.

^c Total number of transformants obtained

^d Transformation frequency represents the number of transformants obtained per 1 μ g of selective plasmid DNA used.

^e Co-transformation is expressed as a percentage.

The number of IL-6 transformants is shown in brackets

The average transformation frequency observed was less than one transformant per μ g of plasmid DNA, being lower than with other fungi transformed using the same selection system (references included in Section 1.1.1) and also transformation of *A. oryzae* using other selection systems (Table 3.1.6).

Table 3.1.6 Frequencies of homologous transformation in *A. oryzae*.

Transfer system	Frequency ^a	Reference
met	20	Iimura et al., 1987
pyrG	40	Ruiter-Jacobs et al., 1989
<i>niaD</i>	64	Unkles et al., 1989b

^a Value expressed in number of transformants per μ g of DNA.

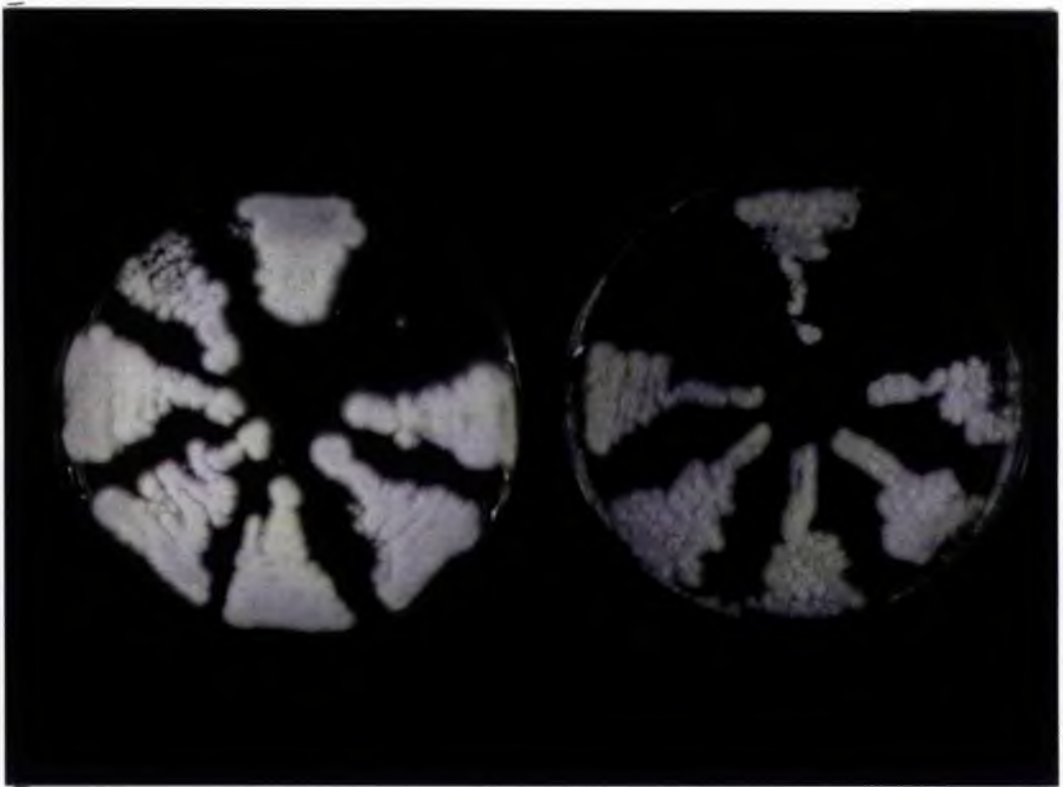
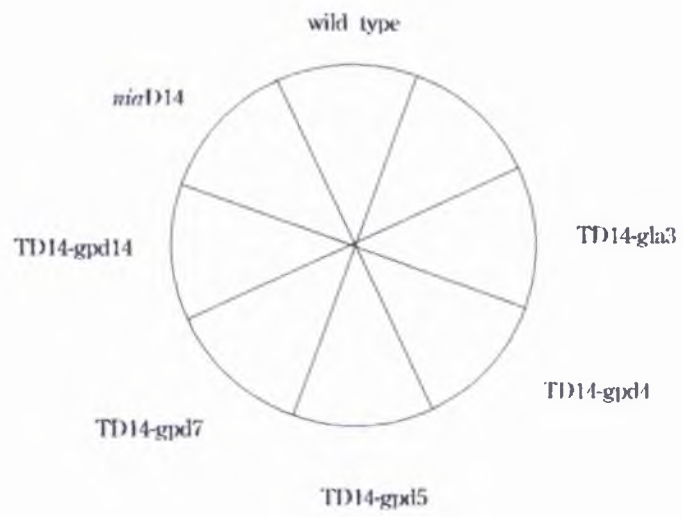
The results showed that the addition of non-selective plasmid to the system increases the transformation frequencies of selective marker. In co-transformation systems using strains *niaD200* and *niaD14*, the frequency of transformation increased two-fold and five-fold using pFGLAhIL6T and pFGPDGLAhIL6 respectively. The percentages of co-transformation were approximately 15 % and 25 % for pFGLAhIL6T and pFGPDGLAhIL6 respectively. The nitrate prototrophic transformants of *A.oryzae niaD* recipient strain are shown in Figure 3.1.4.

10^7 spores of hIL-6 transformants of *A.oryzae* strains *niaD14*, *niaD200* and *niaD500* were inoculated into 100 ml MM containing either 1 % glucose or 1 % maltose as the sole carbon source and ammonium as the sole nitrogen source and incubated at 30°C, for 48 h with agitation. An aliquot of 1 ml of the culture filtrate was frozen in liquid nitrogen and freeze-dried overnight. The resulting powder was resuspended in 50 μ l of sterile distilled water and loaded onto a SDS-polyacrylamide gel.

Two protein bands were observed in SDS-PAGE studies of both recipient and transformant strains which I would like to comment. The first, the major protein produced in *A. oryzae*, α -amylase, has a molecular weight of approximately 51,000 (Hase et al., 1982). The second, a protein band corresponding to a relative molecular mass of 70 kDa whose synthesis was slightly influenced

Figure 3.1.4 Nitrate prototrophic transformants. *A.oryzae niaD14* was co-transformed with pSTA14, as a selective plasmid, and either pFGLAHIL6T (transformant TD14gla3) or pFGPDGLAHIL6 (transformants TD14gpd4, TD14gpd5, TD14gpd7 and TD14gpd14) as non-selective plasmids harbouring hIL-6 cDNA sequence. hIL-6 cDNA sequence is under the control of the *A.niger glaA* or the *A.nidulans gpdA* promoters in plasmids pFGLAHIL6T or pFGPDGLAHIL6 respectively. Transformants were grown on ammonium MM (on the left) and on nitrate MM (on the right).

A. oryzae



by the carbon source. This protein was shown later to be the glycosylated form of glucoamylase from the host strain, *A. oryzae* (Figure 3.1.13). A 100 kDa protein band present in transformants corresponds to the molecular weight of the *A.niger* glucoamylase protein. For identification of the protein band corresponding to hIL-6, it was used yeast recombinant hIL-6 protein as a marker. The *S.cerevisiae* produced hIL-6 is 23 kDa in size and is unglycosylated.

Transformants TD200gla1 (Figure 3.1.5), TD14gla3 (Figure 3.1.6), TD500gla1, TD500gla6, TD500gla10 and TD500gla18 (Figure 3.1.7), which contain the *glaA*/hIL-6 DNA sequences under control of the *A.niger glaA* promoter were shown to secrete *A.niger* glucoamylase in glucose and in maltose media. It is known that the *A.niger glaA* promoter is induced by maltose and repressed by glucose (Shibuya et al., 1990). Nevertheless, it has been reported that integration of multiple copies of the *glaA* gene allows glucoamylase expression even in glucose medium (Macro, 1991 and Contreras et al., 1991). Transformants TD500gpd1, TD500gpd8, TD500gpd15, TD500gpd16, TD500gpd17, TD500gpd18 and TD500gpd21, which contain the *glaA*/hIL-6 DNA sequences under control of the constitutive *A.nidulans gpdA* promoter, does not seem to secrete the recombinant glucoamylase in either glucose or maltose medium (Fig. 3.1.8). It is not clear, first, if these transformants represent false-negative *glaA*/hIL-6 transformants; second, if the expression

Figure 3.1.5

Analysis of transformant TD200glal.

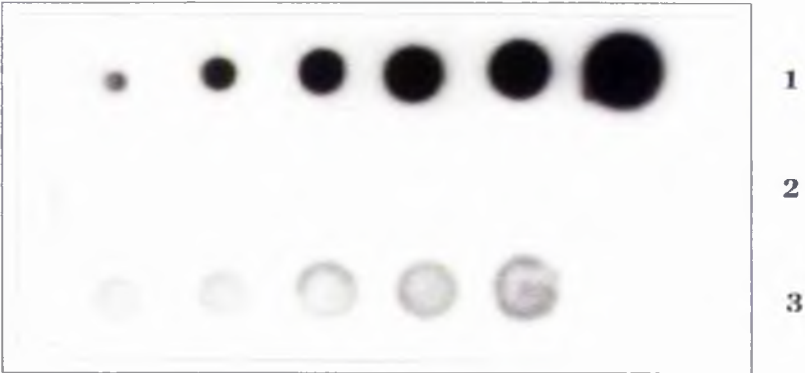
Panel A - Dot blots of genomic DNA. Transformant genomic DNA was dot blotted onto nylon membrane at different concentrations (50, 100, 150, 250, 500 and 1000 mg). Hybridization was carried out at stringent conditions using a 1 kb *EcoRI-HindIII* fragment within hIL-6 cDNA sequence as a probe.

- Line 1 - *A. nidulans* T28, an hIL-6 producer strain
- Line 2 - *A. oryzae* niaD200, recipient strain
- Line 3 - Transformant TD200glal

Panel B - Denaturing polyacrylamide gel electrophoresis of proteins contained in 1 ml of culture filtrate from transformant TD200glal grown in MM containing either 1 % glucose (Lanes 1 and 2) or 1 % maltose (Lanes 3 and 4) as the sole carbon source. The arrows on the right shows the band position of *A.niger* glucoamylase (top) and *A.oryzae* glucoamylase (centre) and amylase (botton).

- Lane M - Molecular weight markers
- Lane C - *S.cerevisiae* recombinant hIL-6
- Lanes 1 and 3 - *A.oryzae* D200, recipient strain
- Lanes 2 and 4 - Transformant TD200glal

A



B

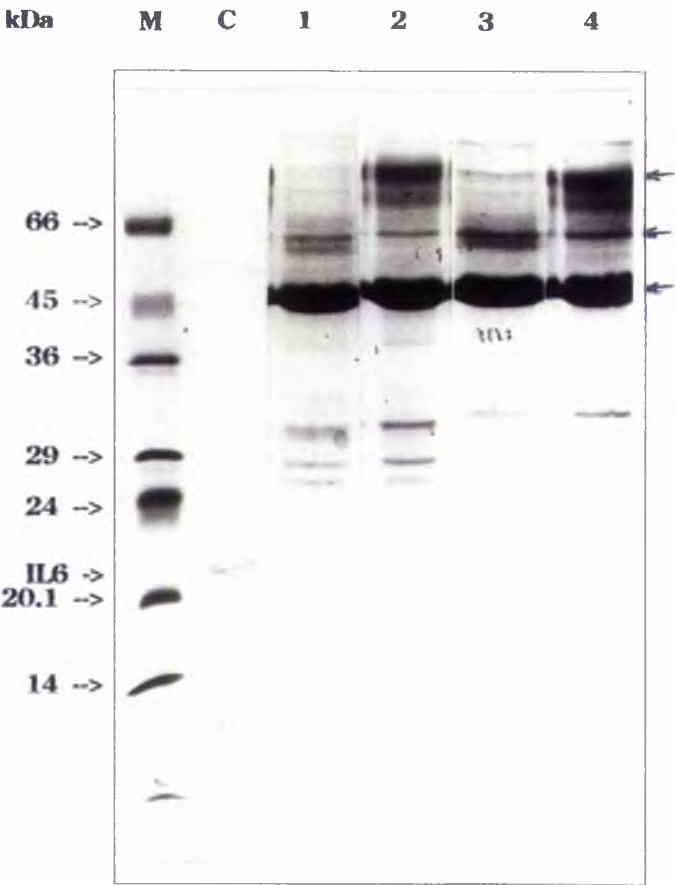


Figure 3.1.6 Studies of transformants from *A.oryzae niaD14* recipient strain.

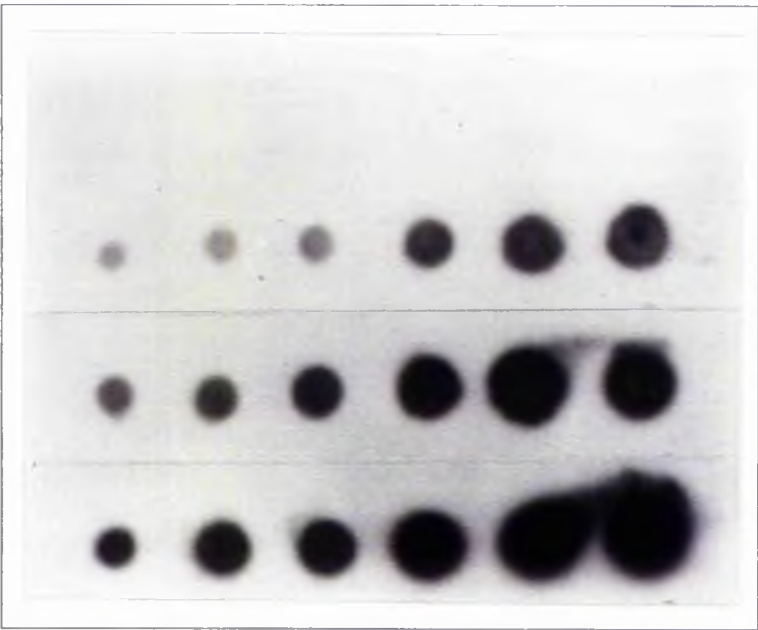
Panel A - Dot blots of genomic DNA. Transformant genomic DNA was dot blotted onto nylon membrane at different concentrations (50, 100, 150, 250, 500 and 1000 mg). Hybridization was carried out at stringent condition using a 1 kb *EcoRI-HindIII* fragment within hIL-6 cDNA sequence as a probe.

- Line 1 - *A. oryzae niaD14*, recipient strain.
- Line 2 - Transformant TD14gla3
- Line 3 - Transformant TD14gpd5
- Line 4 - Transformant TD14gpd14

Panel B - Denaturing polyacrylamide gel electrophoresis of proteins contained in 1 ml of culture filtrate from transformants grown in MM containing either 1 % glucose as the sole carbon source (Lanes 1 to 4) or 1 % maltose (Lanes 5 to 8). The arrow on the right shows the *A.niger* glucoamylase band position.

- Lane C - *S.cerevisiae* recombinant hIL-6
- Lane M - Molecular weight markers
- Lanes 1 and 5 - *A.oryzae niaD14*, recipient strain
- Lanes 2 and 6 - Transformant D14gla3
- Lanes 3 and 7 - Transformant D14gpd5
- Lanes 4 and 8 - Transformant D14gpd14

A



B

M C 1 2 3 4 5 6 7 8

kDa

66 →
45 →
36 →
29 →
24 →
IL6 →
20.1 →
14.2 →

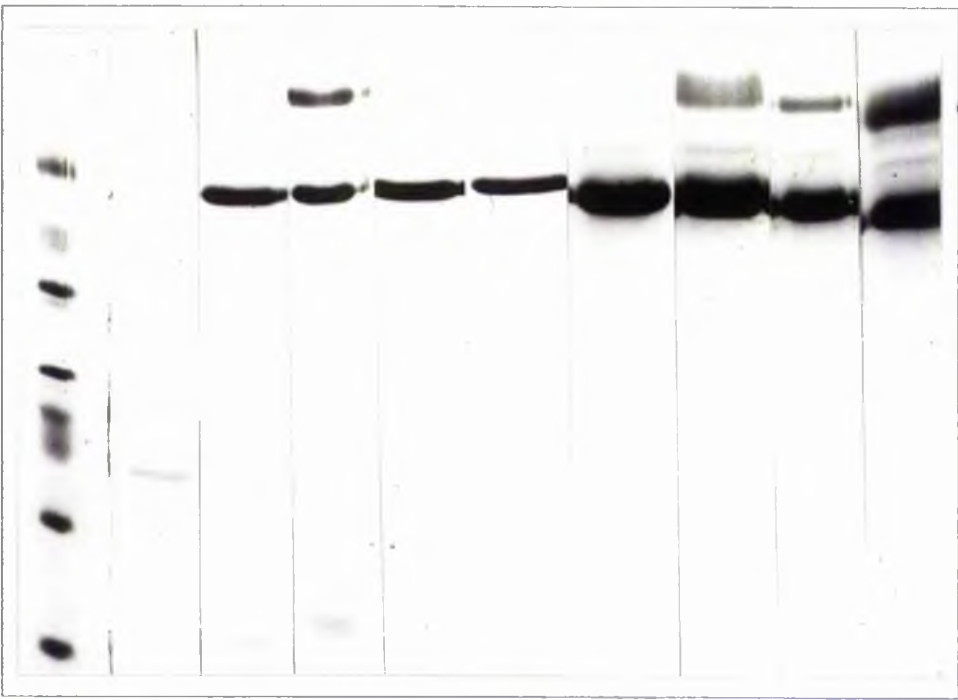


Figure 3.1.7 Studies of transformants from *A.sojae niaD500* recipient strain. SDS-polyacrylamide gel electrophoresis of proteins contained in 1 ml of culture filtrate from transformants grown in MM containing 1 % glucose as the sole carbon source (Panel A) or 1 % maltose as the sole carbon source (Panel B). All the following transformants contain the expression cassette under the control of the *A.niger glaA* promoter. The arrow on the right shows the *A.niger* glucoamylase band position.

Lane M - Molecular weight markers
Lane C - *S.cerevisiae* recombinant hIL-6
Lane 1 - *A.sojae niaD500*, recipient strain
Lane 2 - Transformant TD500gla1
Lane 3 - Transformant TD500gla6
Lane 4 - Transformant TD500gla10
Lane 5 - Transformant TD500gla18

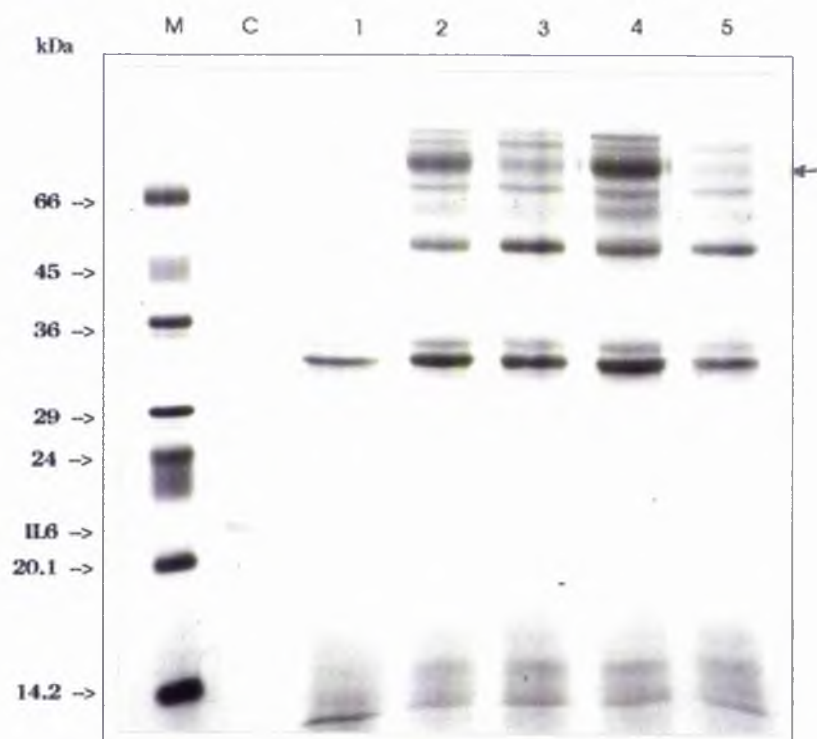
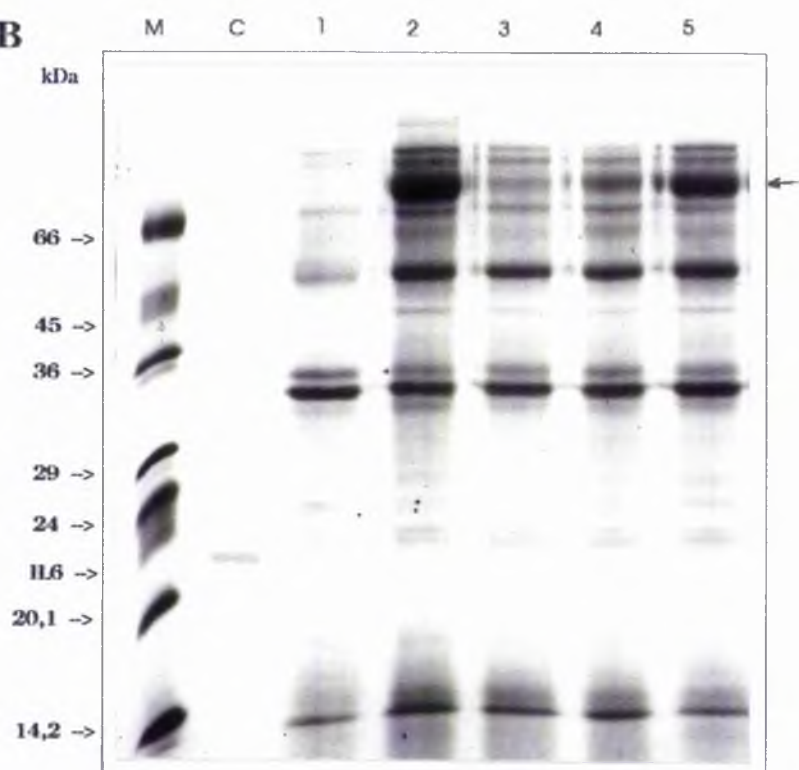
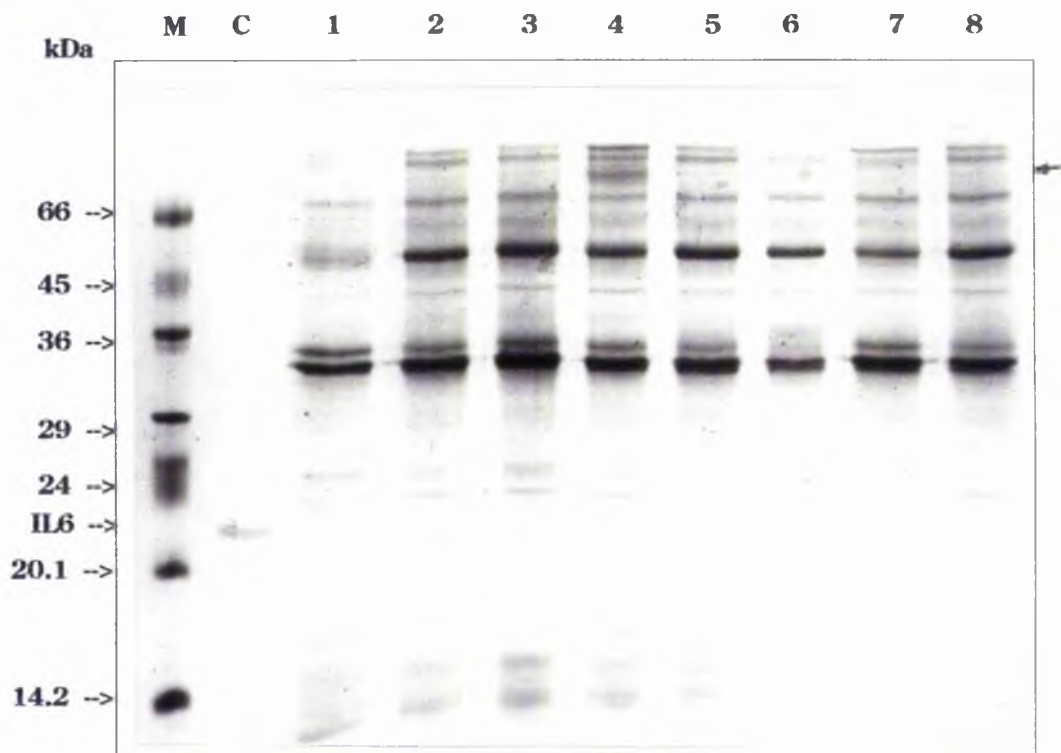
A**B**

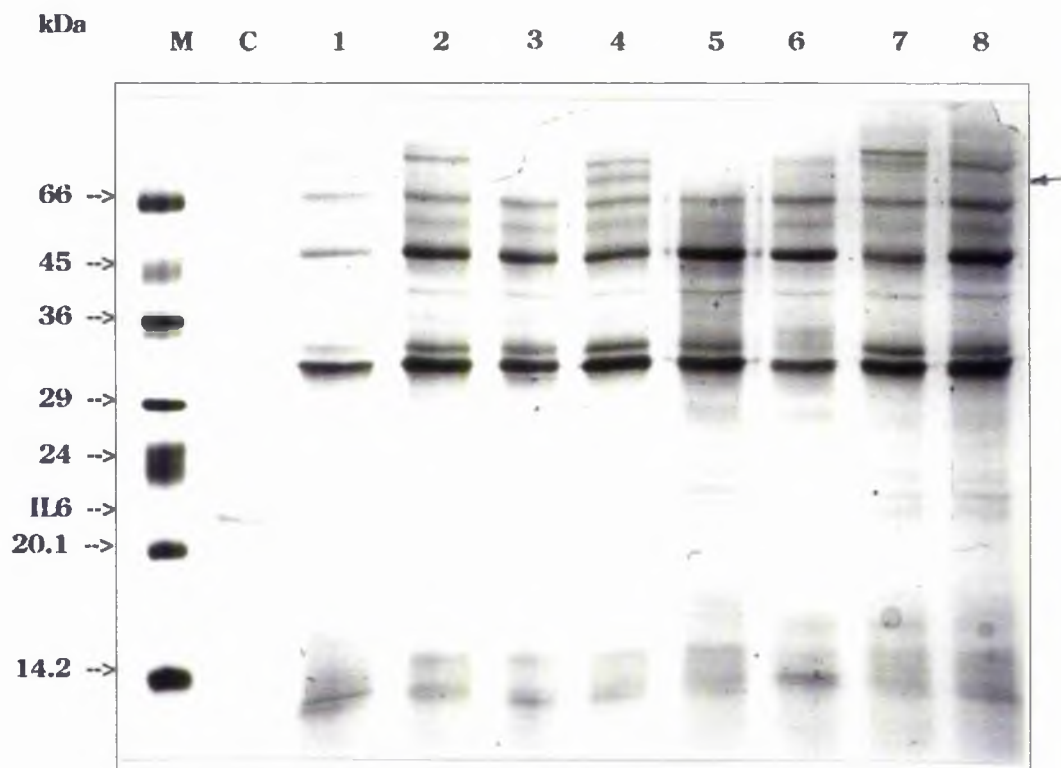
Figure 3.1.8 Studies of transformants from *A. sojae niaD500* recipient strain. SDS-polyacrylamide gel electrophoresis of proteins contained in 1 ml of culture filtrate from transformants grown in MM containing 1 % glucose as the sole carbon source (Panel A) or 1 % maltose (Panel B). All the following transformants contain the expression cassette under the control of the *A.nidulans gpdA* promoter. The arrow on the right shows the *A.niger* glucoamylase band position.

Lane M - Molecular weight markers
Lane C - *S.cerevisiae* recombinant hIL-6
Lane 1 - *A. sojae niaD500*, recipient strain
Lane 2 - Transformant TD500gpd1
Lane 3 - Transformant TD500gpd8
Lane 4 - Transformant TD500gpd15
Lane 5 - Transformant TD500gpd16
Lane 6 - Transformant TD500gpd17
Lane 7 - Transformant TD500gpd18
Lane 8 - Transformant TD500gpd21

A



B



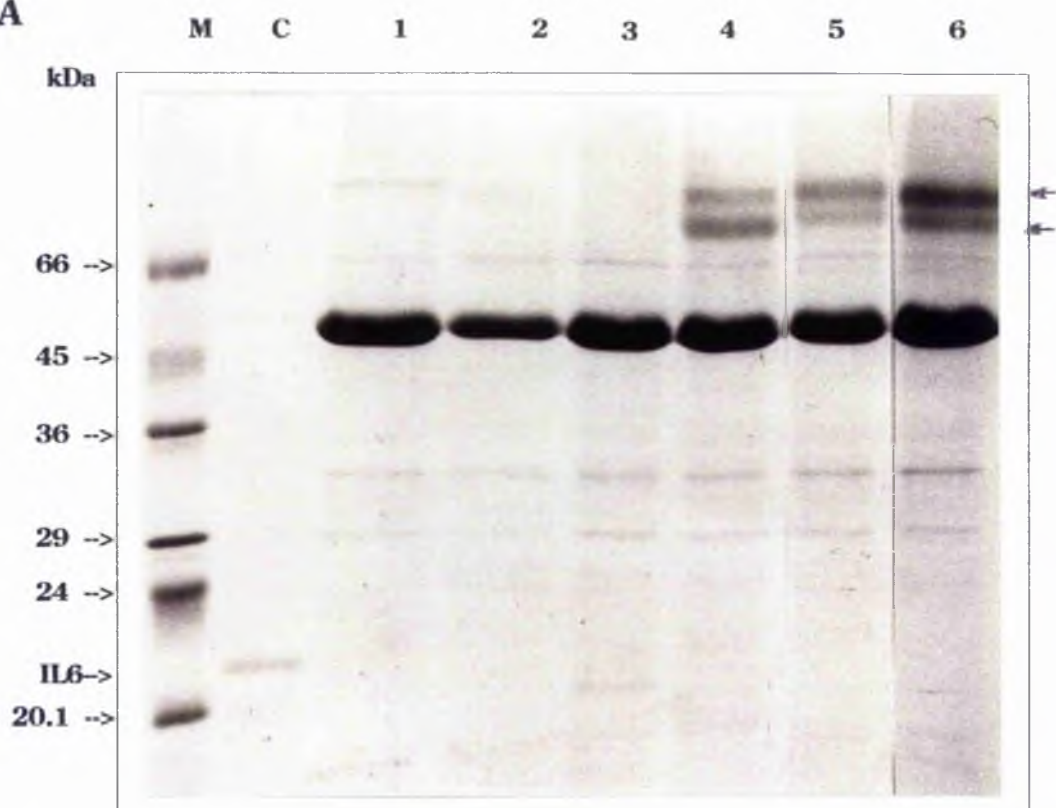
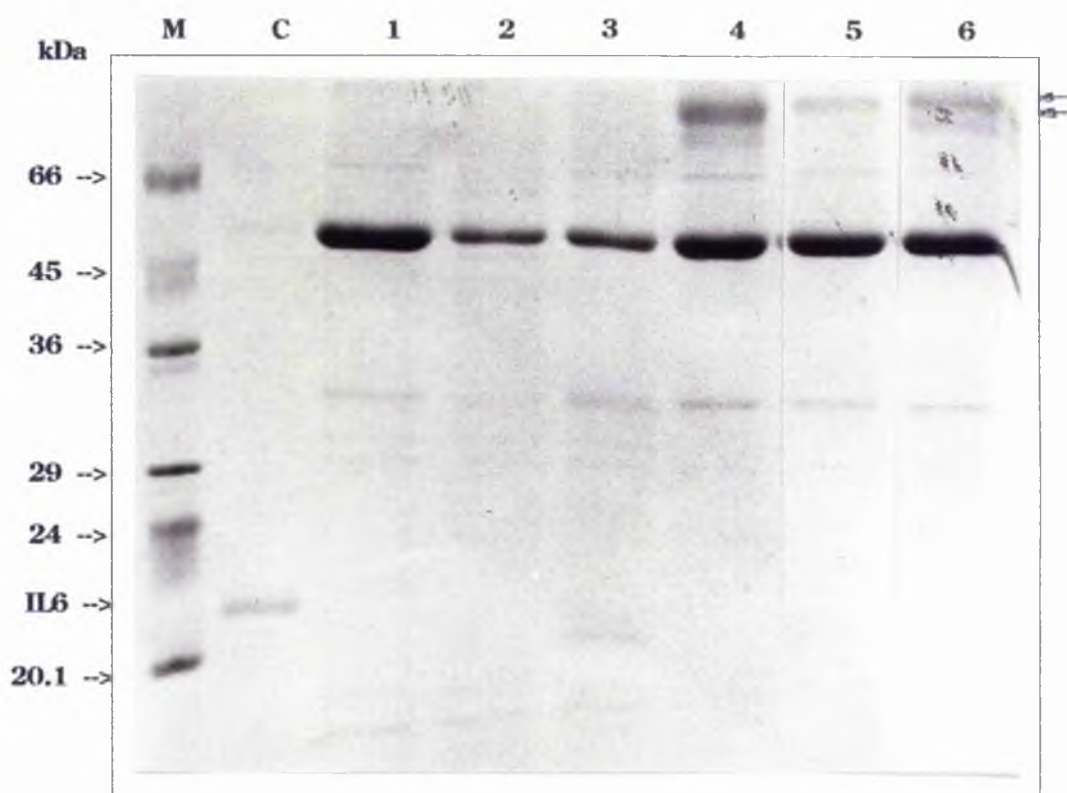
cassettes were damaged by deletion or recombination during the process of integration and third, if the site of integration was not favourable to gene expression. Transformants TD14gpd5 and TD14gpd14, which also contain the *glaA*/hIL-6 DNA sequences under control of the constitutive *A.nidulans* *gpdA* promoter, appeared to secrete the 100 kDa protein only when cells were cultivated in maltose MM (Figure 3.1.6). It was unlikely that the synthesis of glucoamylase was inhibited by glucose, since the expression of glucoamylase in these transformants is under control of the constitutive *gpdA* promoter.

Unfortunately, no single band was visualized on SDS-PAGE that suggested the free form of hIL-6 was present.

To examine the influence of the medium on hIL-6 expression, transformants TD14gla3, TD14gpd5 and TD14gpd14 were grown in CM containing either 1 % glucose or 1 % maltose. Separation of proteins contained in 1 ml of culture filtrate showed two distinct protein bands migrating with a relative mobility of approximately 100 kDa (Figure 3.1.9). These protein band-encoding gene(s) were subject to carbon regulation. The first hypothesis was that these bands could represent different forms of the fused glucoamylase/hIL-6 protein. However, it was unlikely that *A.oryzae* proteases do not recognize the KEX2-site, since KEX2-like endopeptidase has been found in most organisms, such as yeast, several

Figure 3.1.9 Studies of transformants from *A.oryzae niaD14* recipient strain. SDS-polyacrylamide gel electrophoresis of proteins contained in 1 ml of culture filtrates from transformants grown in CM containing 1 % glucose (Panel B) or 1 % maltose (Panel A) as the sole carbon source. Arrows on the right show the *A.niger* glucoamylase band position.

Lane M - Molecular weight markers
Lane C - *S.cerevisiae* recombinant hIL-6
Lane 1 - *A.oryzae niaD14*, recipient strain
Lane 2 - *A.oryzae T2*, IL-6 producer strain
Lane 3 - *A.oryzae T3*, IL-6 producer strain
Lane 4 - Transformant TD14gla3
Lane 5 - Transformant TD14gpd5
Lane 6 - Transformant TD14gpd14

A**B**

filamentous fungi and mammalian cells (Fuller *et al.*, 1988; Contreras *et al.*, 1991; Brennan *et al.*, 1990; Benjannet *et al.*, 1991 and Barr, 1991). Western blot studies of these gels probed with anti-hIL6 pAb and anti-IgG linked to alkaline-phosphatase failed to detect hIL-6 protein in the filtrates of transformant cultures (Lucena, unpublished). Thus, these two protein bands would not represent a fusion GLAA/hIL6 protein but two different carbon inducible proteins or the two forms of *A.niger* glucoamylase.

1.1.1.3 Co-transformation using pAN56-3 or pAN56-4

In order to study the influence of the KEX2-motif in the construction, other hIL-6 containing plasmids with and without KEX2-encoding DNA, respectively pAN56-4 and pAN56-3, were used to transform *A.oryzae* STAN *niaD14* strain.

Protoplasts prepared from an overnight culture of the *niaD14* *A.oryzae* strain were transformed by pSTA14 selective plasmid and either pAN56-3 or pAN56-4. The amount of selective plasmid used was 2.0 μ g and the molar concentration of non-selective plasmids was six-fold higher than the selective one. Selection was carried out by using nitrate as the sole nitrogen source. The transformation frequency obtained was 10 transformants/ μ g of selective DNA used in the transformation reaction (Table 3.1.7).

Table 3.1.7 Transformation frequency of *A.oryzae* STAN *niaD14* strain using *niaD* selection system.

Plasmid used	Number ^a transformants	Transformation ^b frequency	Cotransformation ^c frequency
pSTA14	20	10	NA
pSTA14 + pAN56-3	36	18.5	8 (3)
pSTA14 + pAN56-4	31	15.5	13 (4)

NA Not applicable

^a Total number of transformants obtained

^b Transformation frequency represents the number of transformants obtained per 1 μ g of selective plasmid DNA used.

^c Co-transformation is expressed as a percentage. The number of IL-6 transformants is in brackets.

Transformants were screened by hybridization of genomic DNA dot blot using the 1 kb *EcoRI-HindIII* fragment of hIL-6 gene as a probe. Approximately 10 % of transformants showed positive hybridization. Transformants which do not contain a KEX2-encoding DNA on the plasmid construction were designated TD14-3X and transformants which do contain a KEX2-encoding DNA between the *A.niger glaA* gene and hIL-6 cDNA sequence were designated TD14-4X, where X represents the transformant number (Figure 3.1.10). Transformants TD14-321, TD14-325, TD14-421, and TD14-431 were grown overnight in MM supplemented with 1 % glucose or 1 % maltose as the sole carbon source. An aliquot of 1 ml of culture filtrate was frozen in liquid nitrogen and lyophilized overnight. The resulting powder was resuspended in 50 μ l distilled water and subjected to a SDS-PAGE for protein separation (Fig. 3.1.11). *A.niger*

Figure 3.1.10 Nitrate prototrophic transformants. *A.oryzae* *niaD14* was co-transformed with pSTA14, as a selective plasmid, and either pAN56-3 (transformants TD14-3X) or pAN56-4 (transformants TD14-4X), as non-selective plasmids. Both non-selective plasmids harbouring the *glaA*/*hIL6* DNA sequence under the control of the *A.nidulans* *gpdA* promoter. The pAN56-3 do not contain KEX2-site between the *glaA* and *hIL-6* DNA sequence, while pAN56-4 do contain. Transformants were grown on nitrate MM (on the left side) and on ammonium MM (on the right side).

A. oryzae

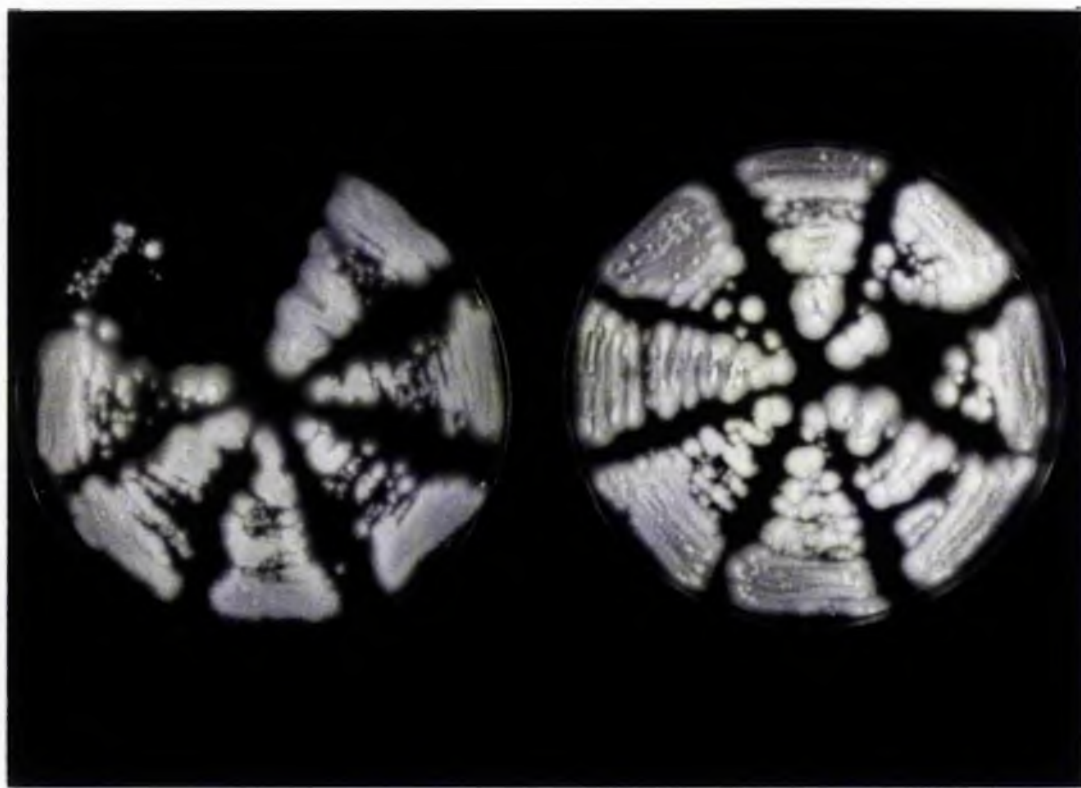
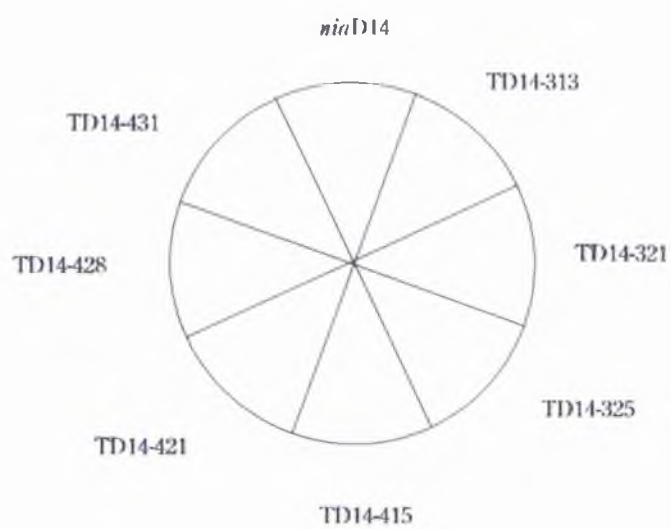


Figure 3.1.11 Studies of transformants from *A.oryzae niaD14* recipient strain.

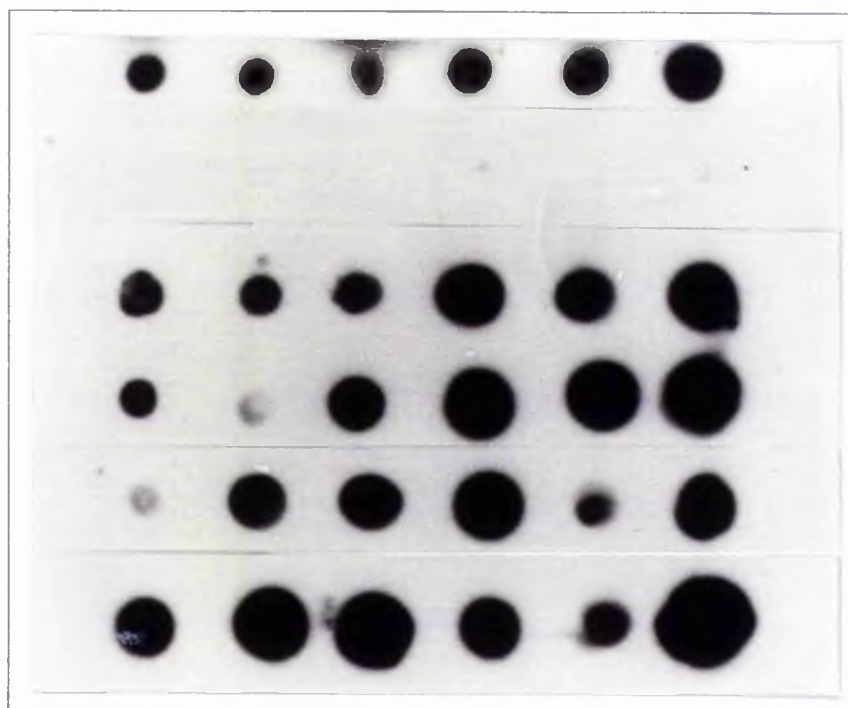
Panel A - Dot blots of genomic DNA. Transformant genomic DNA was dot blotted onto nylon membrane at different concentrations (50, 100, 150, 250, 500 and 1000 mg). Hybridization was carried out at strigent condition using a 1 kb *EcoRI-HindIII* fragment within hIL-6 cDNA sequence as a probe.

- Line 1 - *A. nidulans* T28, an hIL-6 producer strain
- Line 2 - *A. oryzae niaD14*, recipient strain
- Line 3 - Transformant TD14-315
- Line 4 - Transformant TD14-321
- Line 5 - Transformant TD14-421
- Line 6 - Transformant TD14-431

Panel B - Denaturing polyacrylamide gel electrophoresis of proteins contained in 1 ml of culture filtrates of transformants grown in MM containing either 1 % glucose (Lanes 1 to 5) or 1 % maltose (Lanes 6 to 10) as the sole carbon source. The arrow on the right shows the *A.niger* glucoamylase band position.

- Lane M - Molecular weight markers
- Lane C - *S.cerevisiae* recombinant hIL-6
- Lanes 1 and 6 - *A.oryzae niaD14*, recipient strain
- Lanes 2 and 7 - Transformant TD14-315
- Lanes 3 and 8 - Transformant TD14-321
- Lanes 4 and 9 - Transformant TD14-421
- Lanes 5 and 10 - Transformant TD14-431

A



<-- 1

<-- 2

<-- 3

<-- 4

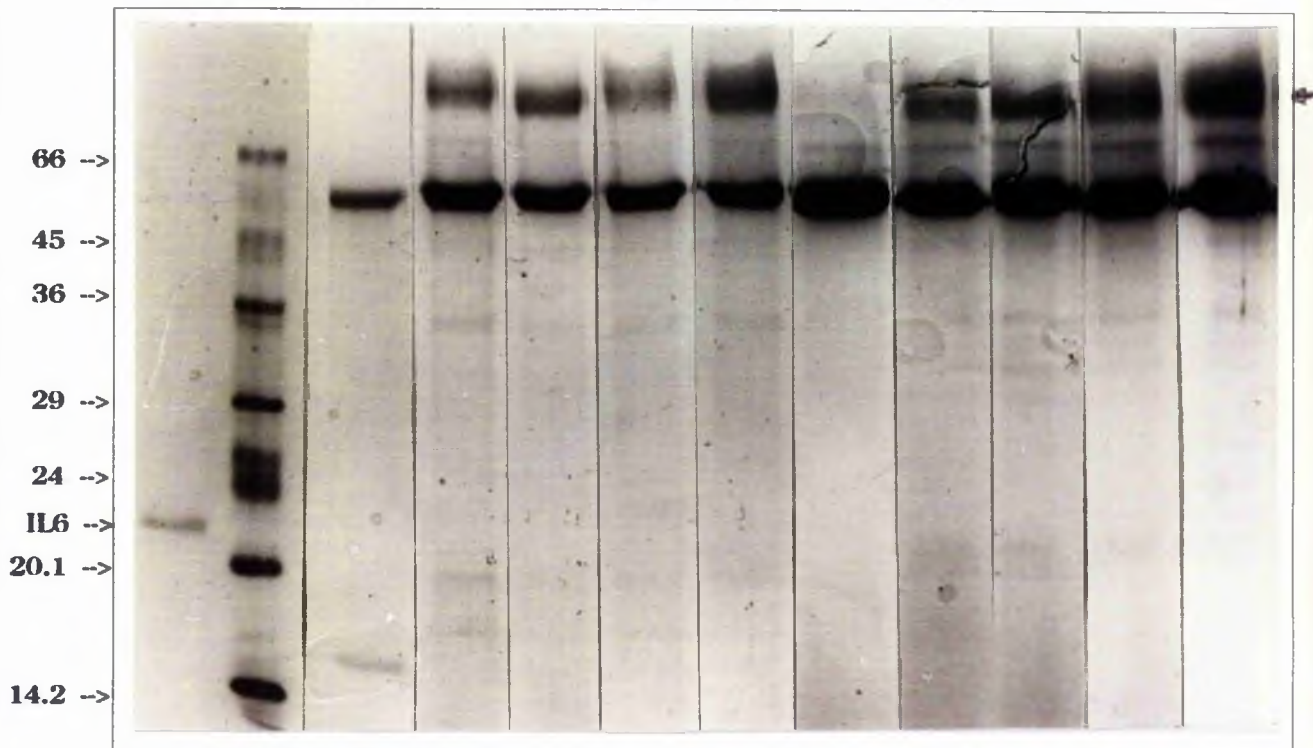
<-- 5

<-- 6

B

kDa

C M 1 2 3 4 5 6 7 8 9 10



glucoamylase (100 kD) protein band was present in these transformants. This band is not inducible by maltose, since the *A.niger glaA* gene in either pAN56-3 or pAN56-4 is under control of the *A.nidulans gpdA* promoter. The *A.oryzae* glucoamylase (68 kDa) and α -amylase (55 kDa) protein bands were also identified. Unfortunately, no band was detected on SDS-PAGE gel which suggested the presence of a free form of the hIL-6 protein.

Immunological reactions were carried out as described in the Materials and Methods 2.8.3.1, using a anti- hIL6 pAb. Transformants TD14-321 and TD14-325 produced a fused Glucoamylase/hIL-6 protein as expected, since the constructs did not have a KEX2-encoding DNA. Transformants containing a KEX2-encoding DNA did not react with the anti-hIL6 pAb (Figure 3.1.12). This fact suggested: First, *A. oryzae* recognized the KEX2-site as other *Aspergillus* species. Second, proteases secreted into the medium might have been responsible in the failure to detect the free form of hIL-6 protein. Thus, hIL-6 might have been produced and secreted but easily degraded by proteases in the medium. However, the reason that the *A.niger* glucoamylase were more resistant to proteases action might be due to its homology of 67 % in the deduced amino acid sequence with the *A. oryzae* glucoamylase (Hata et al., 1991). Third, probably, the wide protein bands with relative mobility of approximately 100 kDa detected on SDS-PAGE of transformants represent the different glycosylation

Figure 3.1.12 Studies of transformants from *A.oryzae niaD14* recipient strain. Transformants were grown in MM containing 1 % maltose as the sole carbon source. Proteins contained in 1 ml of culture filtrate were transferred onto nitrocellulose membrane and probed with anti-hIL6 pAB and anti-rabbit IgG conjugated to alkaline phosphatase. hIL-6 was detected by a enzymatic method (see Materials and Methods for details).

Lane M - Molecular weight markers
Lane C - *S.cerevisiae* recombinant hIL-6
Lane 1 - *A.oryzae* wild type
Lane 2 - *A.oryzae niaD14*, recipient strain
Lane 3 - Transformant TD14-315
Lane 4 - Transformant TD14-321
Lane 5 - Transformant TD14-421
Lane 6 - Transformant TD14-431

kDa M C 1 2 3 4 5 6 7



patterns from both forms of the *A. niger* glucoamylase (Figures 3.1.9 and 3.1.11). To test this hypothesis, the *A.oryzae niaD14* strain was co-transformed with pANG3 which encodes the complete *A.niger glaA* gene. Proteins contained in 1 ml of culture filtrate of transformant TD14-G3 were western blotted and probed with anti-*A.niger* GLAA pAb. The results revealed a wide band migrating at the same rate as those wide bands detected in the SDS-PAGE gel of hIL-6 transformed strains (Figure 3.1.13). Finally, it seemed that the culture conditions influence the expression of each of these forms, as occurred when TD14gla3, TD14gpd5 and TD14gpd14 transformats were grown in minimum and complete medium (Figures 3.1.6 and 3.1.9).

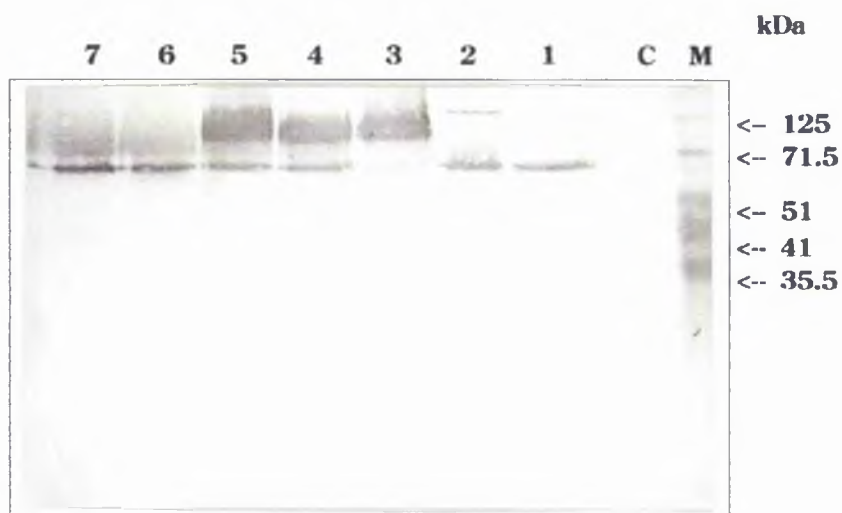
1.1.2 The *argB* selection system

The *argB* gene encodes ornithine transcarbamyl transferase, an essential enzyme in the arginine biosynthetic pathway (Figure 3.1.14). The *A.nidulans argB* gene does not contain introns a fact which represents one less possible barrier to heterologous gene expression. It is also likely to have codon usage similar to most fungal translation systems (Buxton et al., 1985). This system requires a recipient strain carrying a complementary mutation with low reversion frequency. The *arg*⁻ auxotrophic marker results in a very

Figure 3.1.13 Studies of transformants from *A.oryzae niaD14* recipient strain. Proteins contained in 1 ml of culture filtrate of the following transformants were transferred onto nitrocellulose membrane and probed with anti-*A.niger* GLAA pAb (Panel A) or anti-hIL-6 pAb (Panel B) and anti-rabbit IgG linked to alkaline phosphatase.

Lane M - Molecular weight markers
Lane C - *S.cerevisiae* recombinant hIL-6
Lane 1 - *A.oryzae* wild type
Lane 2 - *A.oryzae niaD14*, recipient strain
Lane 3 - Transformant TD14-G3
Lane 4 - Transformant TD14gla3
Lane 5 - Transformant TD14gpd14
Lane 6 - Transformant TD14-325
Lane 7 - Transformant TD14-431

A



B

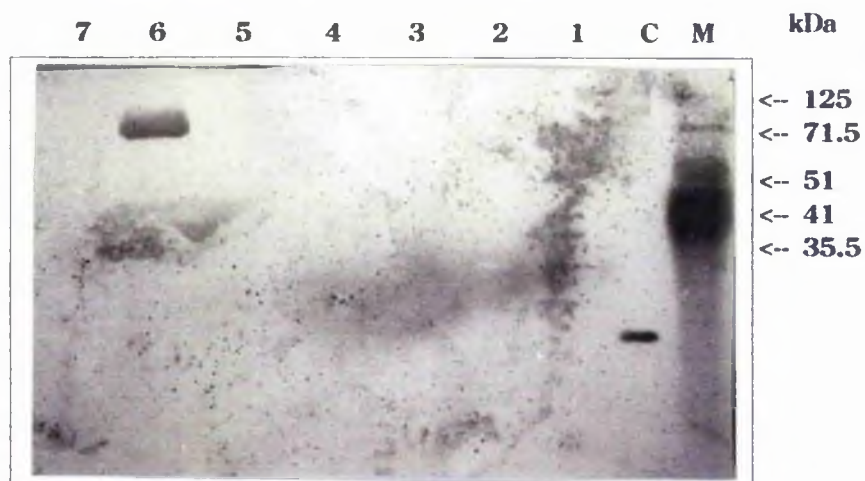
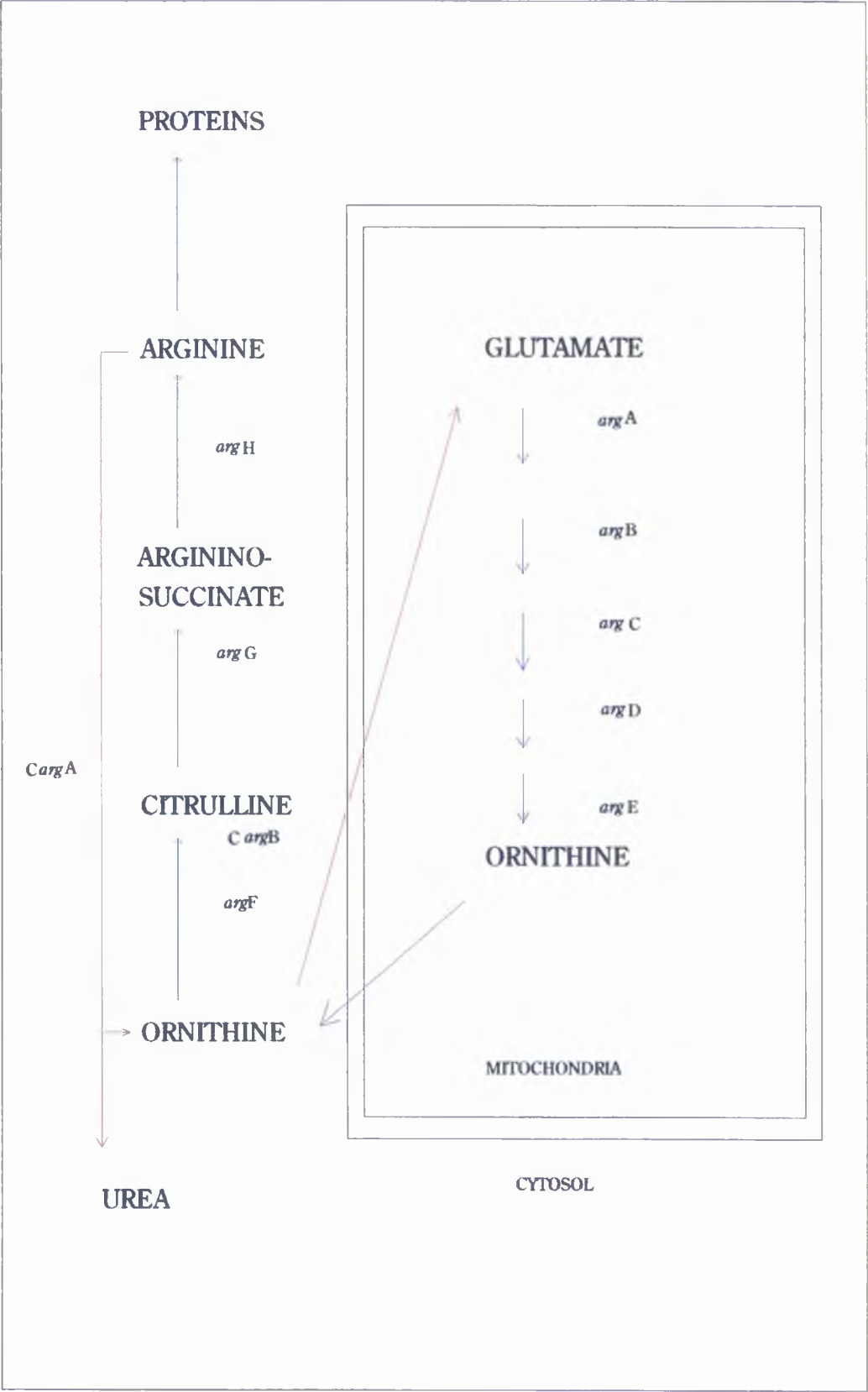


Figure 3.1.14 The biochemistry and genetics of the arginine metabolism in the yeast *S.cerevisiae* (Wiame et al., 1980) and the filamentous fungi *A.nidulans* (Pateman & Kinghorn, 1979). The biosynthesis of L-arginine from L-glutamate is shown in blue line. The conversion of L-glutamate to L-ornithine is carried out in five enzymatic reactions inside the mitochondria. One of these reactions is carried out by ornithine carbamoyl transferase, the *argB* gene product. In the cytosol, the conversion of L-ornithine to L-arginine is performed in three enzymatic reactions. The pathways of L-arginine degradation is shown in red line. Genes involved in arginine catabolism are represented by *Carg*.



clear phenotype thus, complementation by a few copies of *argB* gene is easily detected (Johnstone et al., 1990).

A. oryzae 1560-6 recipient strain carrying an *argB* mutation was transformed with pSTA4 which carries the *A.nidulans argB* gene and either pFGLAhIL6T or pFGPDGLAhIL6. 1.5 μ g of selective plasmid was used and the molar concentration of non-selective plasmid used was six-fold higher than the selective one. The results are shown in Table 3.1.8.

The frequency of 2.67 transformants / μ g of selective DNA using the *argB* selection system is similar to the transformation frequency reported for *A.nidulans* (0.6 to 4 transformants per μ g DNA, Buxton et al., 1985) and higher than that reported for *A.oryzae* (0.7 transformants per μ g DNA, Gomi et al., 1987).

Table 3.1.8 Transformation frequency of *A. oryzae* 1560-6 strain using the *argB* selection system.

plasmid	Number	Frequency ^a	Cotransformation ^b
pSTA4	4	2.67	NA
pSTA4 + pFGLAhIL6T	16	10.67	25 (4)
pSTA4 + pFGPDGLAhIL6	1	0.66	100 (1)

NA Not applicable

^a Transformation frequency represents the number of transformants obtained per 1 μ g of selective plasmid DNA used.

^b Co-transformation is expressed as a percentage. The number of IL-6 transformants is in brackets.

Transformants T1560-gla6, T1560-gla8, T1560-gla9, T1560-gla15 and T1560-gpd1 (Figure 3.1.15) were grown in 100 ml MM containing either 1 % glucose or 1 % maltose as the sole carbon source. The sole nitrogen source used was ammonium in an attempt to repress protease activity (Cohen, 1973a,b; Wiame et al., 1986). Proteins contained in 1 ml of culture filtrate of T1560-gla15 and T1560-gpd1 were separated in SDS-PAGE gel. The gel was stained with Coomassie blue dye and destained with methanol-acetic acid solution.

The results showed a wide band of relative molecular weight of around 100 kDa in both T1560-gla15 and T1560-gpd1 (Figure 3.1.16). However, no hIL-6 corresponding band was suggested in the Coomassie stained gel comparing with the yeast recombinant hIL-6 marker. Western blot analysis were carried out using anti-hIL6 pAb (Figure 3.1.17). This antibody reacted with a band of relative mobility of approximately 120 kDa in transformant T1560-gla15, which suggested that the hIL-6 protein was secreted as a fused protein with glucoamylase. However, what occurred with this particular transformant was not well understood, since *A.oryzae* proteases are able to recognize the KEX2-site (Section 1.1.1.3). One possible explanation could be the construction drove the fused glucoamylase-hIL6 protein into the ER and once inside the ER the protein became folded. However, during the folding, the amino acid sequence flanking the KEX2-motif could have induced

Figure 3.1.15 Arginine prototrophic transformants. *A.oryzae* 1560-6 was co-transformed with pSTA4, as a selective plasmid, and either pFGLAHIL6T (transformants T1560-gla6, T1560-gla8, T1560-gla9 and T1560-gla15) or pFGPDGLAHIL6 (transformant T1560-gpd1), as non-selective plasmids encoding hIL6 cDNA sequence. The hIL-6 cDNA sequence is under the control of the *A.niger glaA* or the *A.nidulans gpdA* promoters in plasmid pFGLAHIL6T or pFGPDGLAHIL6, respectively. Transformants were grown on ammonium MM in absence (on the left side) or presence (on the right side) of arginine.

A. oryzae

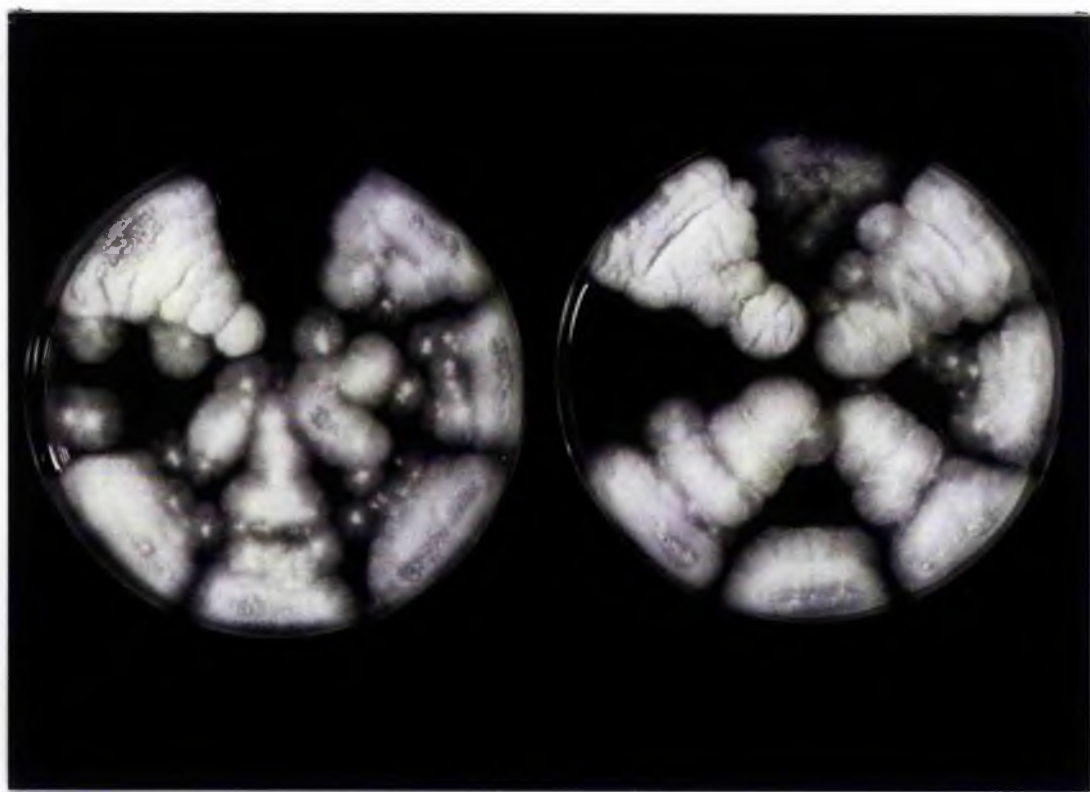
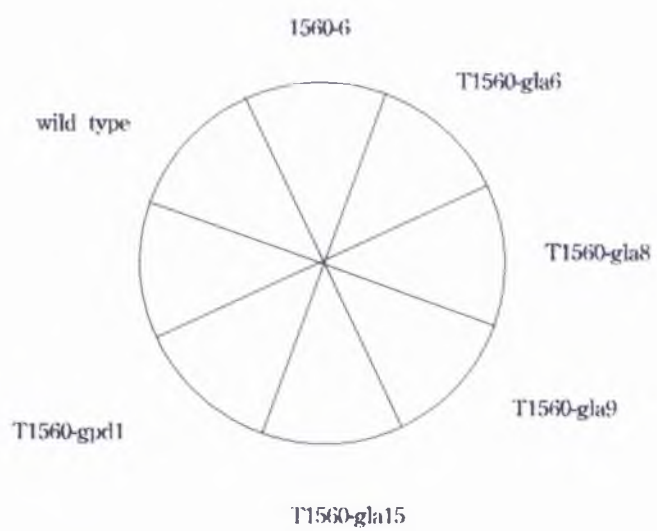


Figure 3.1.16 Studies of transformants from *A.oryzae* 1560-6 recipient strain.

Panel A - Dot blots of genomic DNA. Transformant genomic DNA was dot blotted onto nylon membrane at different concentrations (50, 100, 150, 250, 500 and 1000 mg). Hybridization was carried out at stringent conditions using a 1 kb *EcoRI-HindIII* fragment within hIL-6 cDNA sequence as a probe.

- Line 1 - *A.nidulans* T28, an hIL-6 producer strain
- Line 2 - *A.oryzae* 1560-6, recipient strain
- Line 3 - Transformant T1560-gla15
- Line 4 - Transformant T1560-gpd1

Panel B - Denaturing polyacrylamide gel electrophoresis of proteins contained in 1 ml of culture filtrates of transformants grown in MM containing either 1 % glucose (Lanes 1 to 3) or 1 % maltose (Lanes 4 to 6) as the sole carbon source. The arrow on the right shows the *A.niger* glucoamylase band position.

- Lane M - Molecular weight markers
- Lane C - *S.cerevisiae* recombinant hIL-6
- Lanes 1 and 4 - *A.oryzae* 1560-6, recipient strain
- Lanes 2 and 5 - Transformant T1560-gla15
- Lanes 3 and 6 - Transformant T1560-gpd1

2

3

4

kDa

C

M

1

2

3

4

5

6

66 -->

45 -->

36 -->

29 →

24 -->

IL6 ->

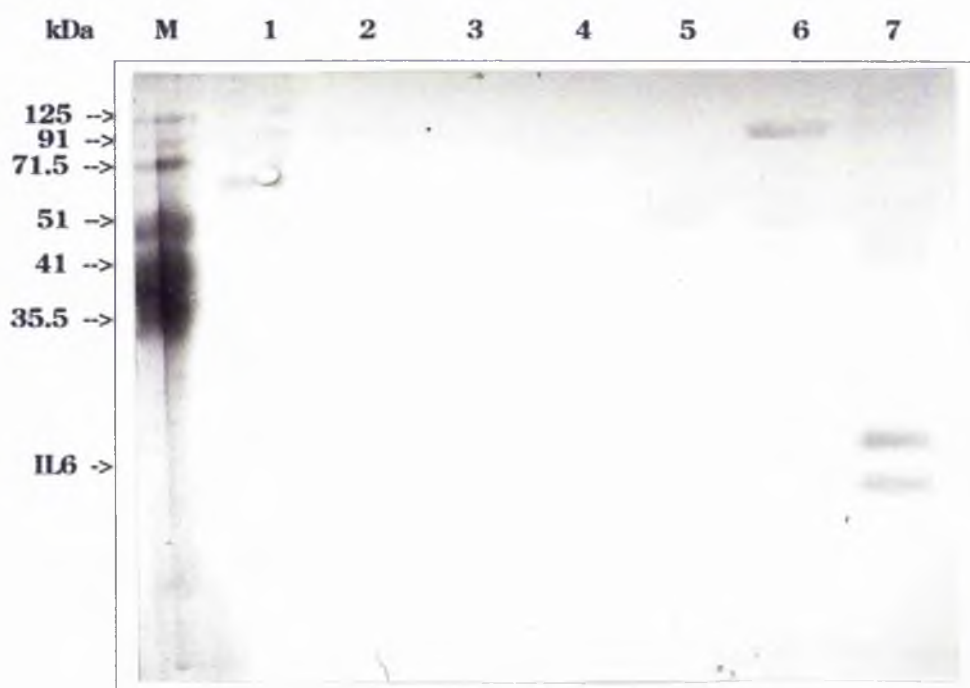
20.1 -->

14.2 ->

—

Figure 3.1.17 Studies of transformants from *A.oryzae* 1560-6 recipient strain. Proteins contained in 1 ml of culture filtrates of the following transformants were transferred onto nitrocellulose membrane and probed with anti-hIL6 pAB and anti-rabbit IgG conjugated to alkaline phosphatase.

Lane M - Molecular weight markers
Lane 1 - *S.cerevisiae* recombinant hIL-6
Lane 2 - *A.oryzae* 1560-6, recipient strain
Lane 3 - Transformant T1560-gla6
Lane 4 - Transformant T1560-gla8
Lane 5 - Transformant T1560-gla9
Lane 6 - Transformant T1560-gla15
Lane 7 - Transformant T1560-gpd1



a molecule conformation which masked the KEX2-site. Thus, the hybrid protein was not cleaved at the KEX2-site and the hIL-6 protein was secreted fused to glucoamylase. However, it was unlikely that the problem was with the construction work at the KEX2-motif since other transformants from previous experiments, for example TD200gla1 and TD14gla3, did not show the same phenomena. Another possible explanation could be that the KEX2-endopeptidase, which has been shown to be a resident protein in the later Golgi compartment in *S.cerevisiae*, or an equivalent *Aspergillus* endopeptidase was saturated by the substrate and then the hybrid GLAA/hIL-6 protein was secreted. In this case, the fused protein seemed to protect hIL-6 from being digested, whereas free hIL-6 in the medium was selectively degraded.

Transformant T1560-gpd1 showed two bands on western blot. The first one has a relative mobility similar to that of the recombinant hIL-6 from *S. cerevisiae* (23 kDa), which is non-glycosylated. The second band migrates faster than the previous one, and may represent a degradation product of secreted hIL-6, or alternatively another form of mature hIL-6 expressed in *A. oryzae*. Mature hIL-6 has been reported to vary in size depending on the producing cell type (Carrez et al., 1990; Gross et al., 1989).

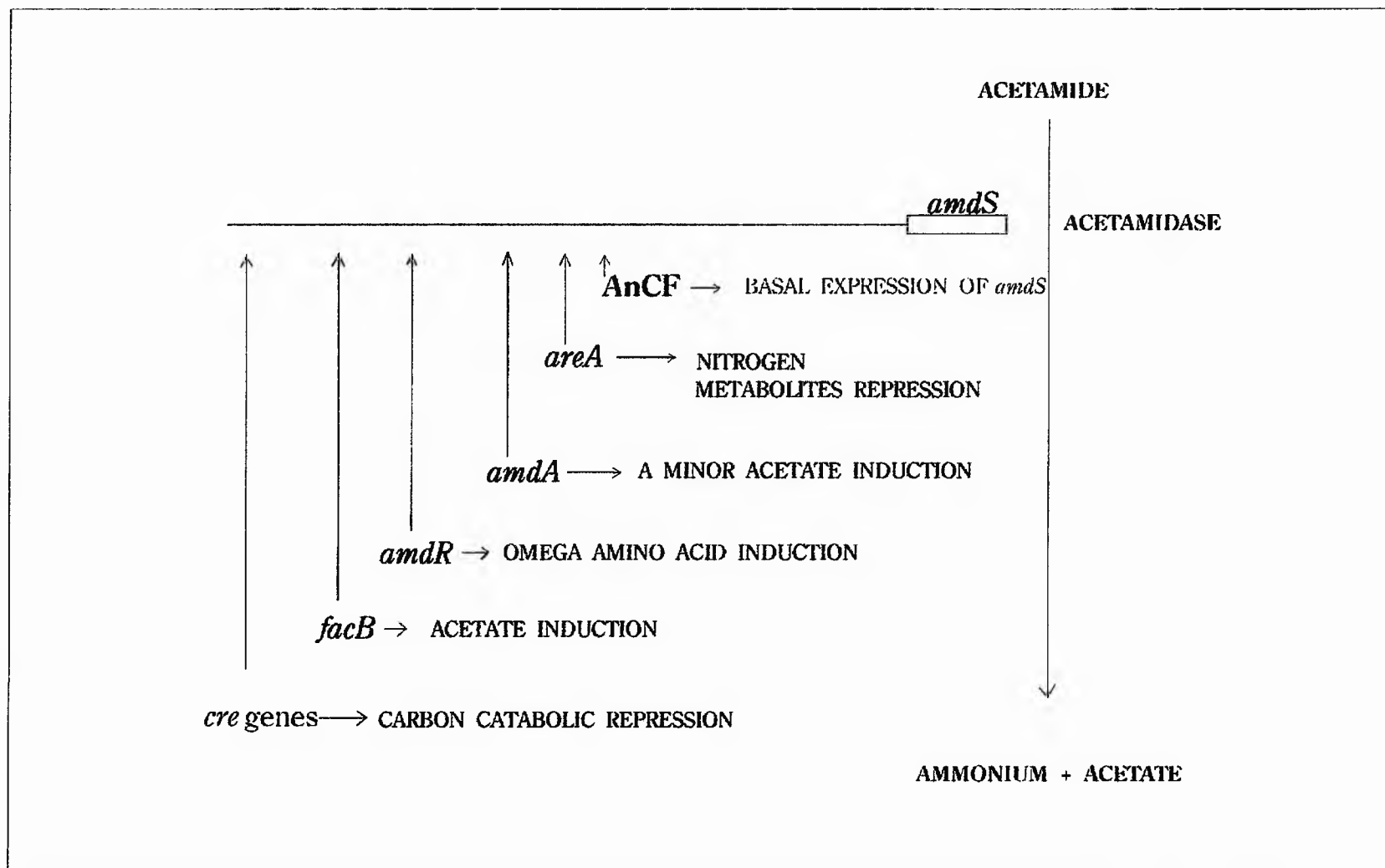
Unfortunately, the hIL-6 level in this transformant was insufficient to be detected on Coomassie stained gel.

1.1.3 The *amdS* selection system

The acetamidase-encoding gene (*amdS*), a dominant selectable marker, allows acetamide to be used as the nitrogen and / or carbon source (Bailey & Arst, 1975). The acetamidase enzyme converts acetamide to acetate and ammonium. Isolation of *A.nidulans amdS* mutants have been possible by selection of fluoro-acetate resistance (Hynes & Davis, 1986). The importance of the *amdS* gene is yet unknown. However, multiple regulatory signals have been identified on *A.nidulans amdS* promoter (Figure 3.1.18) (Hynes & Davis, 1986; Kelly & Hynes, 1987; Hynes et al., 1988; van Heeswijck & Hynes, 1991). These *cis*-acting sites are recognized by the product of *trans*-acting regulatory genes which, therefore, control the *amdS* expression. For instance, the AnCF factor has been found to be responsible for the basal expression of *amdS* gene. The *amdS* expression is induced by AMDR (omega amino acid induction), AMDA (minor acetate induction) and FACB (acetate induction) proteins. The products of *areA* and *creA* genes cause nitrogen metabolite and carbon catabolite repression (for a review see Hynes, 1993).

Plasmids containing the *amdS* gene have been used to develop a transformation system for *A. nidulans*

Figure 3.1.18 The biochemistry and genetic regulation of acetamide utilization in *A.nidulans* (Andrianopoulos & Hynes, 1988; Hynes & Andrianopoulos, 1989; Hynes, 1993). See text for details.



(Tilburn et al., 1983), *A. niger* (Kelly & Hynes, 1985), *A. oryzae* (Christensen et al., 1988 and Gomi et al., 1991), *Penicillium chrysogenum* (Beri and Turner, 1987) and *Trichoderma reesei* (Penttila et al., 1987).

The acetamidase system has permitted high frequency of transformation, with multiple copies integrated. Furthermore, co-transformation of an unselected plasmid using p3SR2 (containing the *amdS* selective marker) is relatively high (Kelly & Hynes, 1985).

It has been reported that transformation of a strain which grows very poorly on acetamide shows high background, due to degradation of acetamide by non-specific amidases and impurities in the agar (Tilburn et al., 1983). However, the addition of caesium chloride to solid medium reduces this problem. Thus, *amdS*⁺ transformants can be selected by strong growth, whereas the weaker colonies consist of abortive transformants.

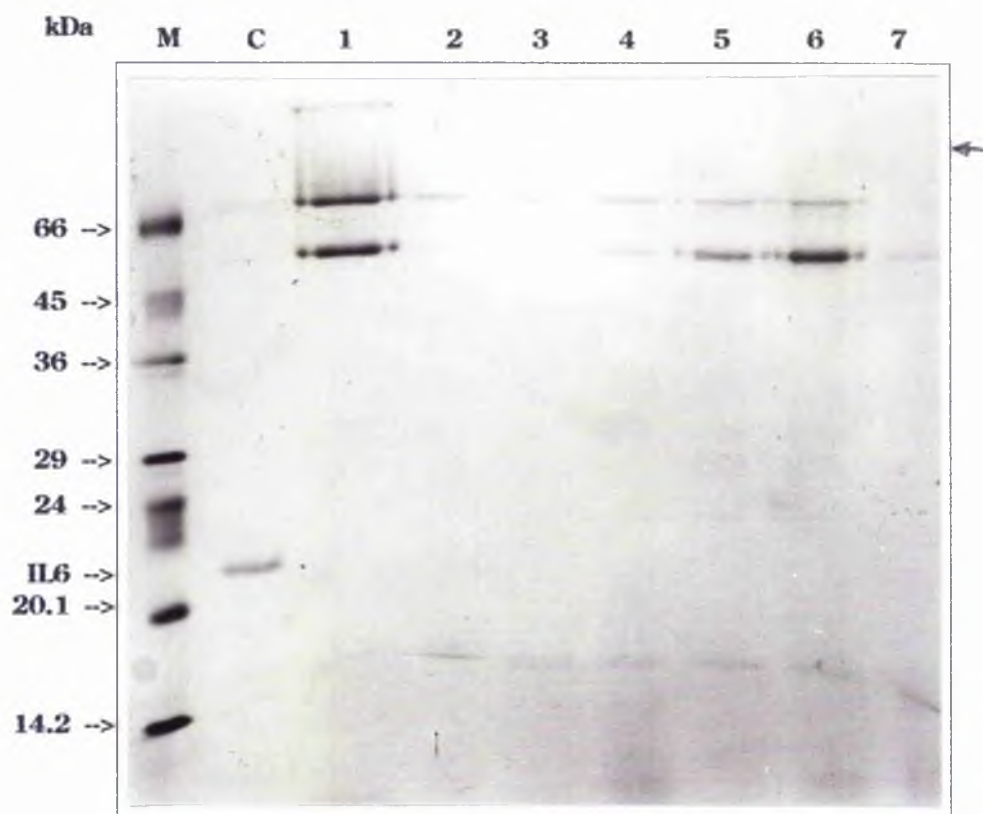
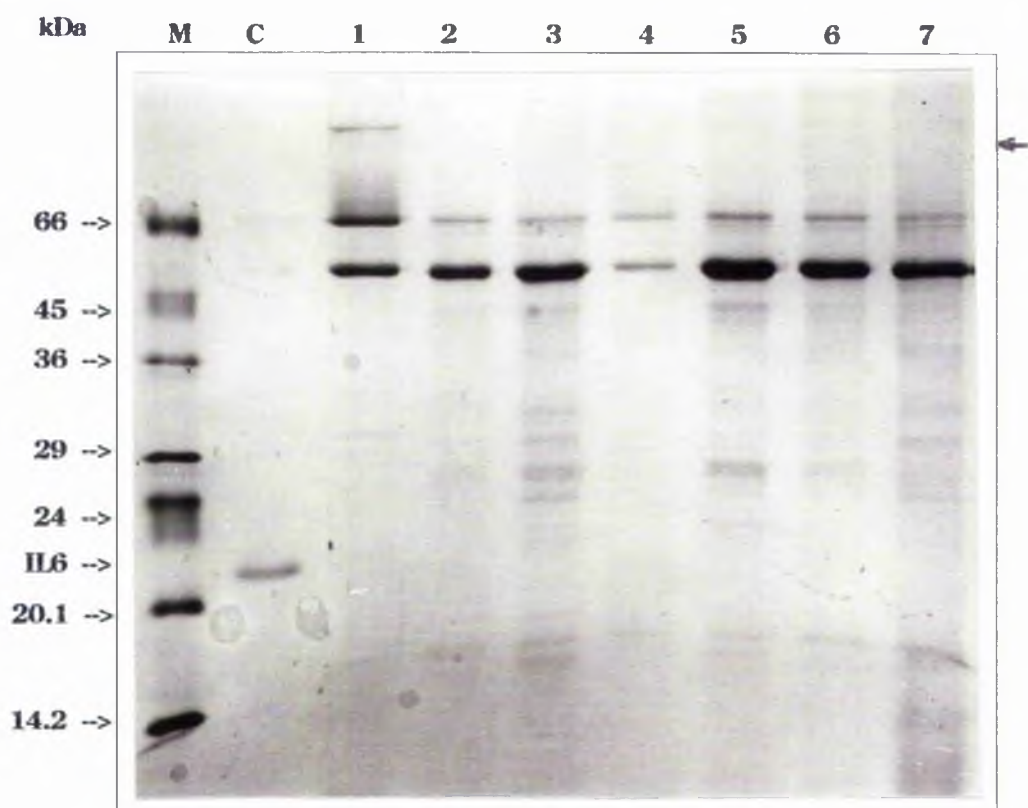
In an attempt to obtain high levels of hIL-6 production by increasing the copy number of the hIL-6 gene integrated into the fungal genome, the *A.oryzae* 1560-6 industrial strain was transformed using *amdS* selection system. *A.oryzae* strain 1560-6 shows a poor growth on MM containing 2 mM acetamide as the sole nitrogen and carbon sources. Protoplasts prepared as described in Materials and Methods were transformed with p3RS2 and either pFGLAhIL6T or pFGPDGLAhIL6. 1.5 µg of

selective plasmid was used and the molar concentration of non-selective plasmids used was six-fold higher than the selective one (i.e. p3SR2). In the first instance, 5 mM caesium chloride was added to the selective medium (MM containing 2 mM acetamide as the sole nitrogen source), however, the background was too high to distinguish transformants. Then transformation was carried out using 10 and 15 mM caesium chloride in the selective medium. The background was significantly reduced in the 15 mM caesium chloride-containing selective medium.

A large number of transformants was obtained, although only a few transformed colonies were slightly larger and showed signs of sporulation after 10 days of incubation. These transformants were screening by hybridization of dot blots of genomic DNA using the *EcoRI-HindIII* fragment of the hIL-6 cDNA sequence as a probe. Three of pFGLAhIL6T transformed strains and three of pFGPDGLAhIL6T transformed strains were selected and inoculated into MM supplemented with 1 % glucose or 1 % maltose as the sole carbon source and 10 mM ammonium as the sole nitrogen source and incubated with agitation, at 30°C, for 48 h. Proteins contained in 1 ml of culture filtrate were analysed by SDS-PAGE. No bands were identified, by size comparison, as being either the free form of hIL-6 or the *A.niger* glucoamylase protein (Figure 3.1.19). Western blots probed with anti-hIL6 or

Figure 3.1.19 Studies of transformants from *A.oryzae* 1560-6 recipient strain. SDS-polyacrylamide gel electrophoresis of proteins contained in 1 ml of culture filtrates of transformants grown in MM containing 1 % glucose (Panel A) or 1 % maltose (Panel B) as the sole carbon source. The arrow on the right shows the *A.niger* glucoamylase band position.

Lane M - Molecular weight markers
Lane C - *S.cerevisiae* recombinant hIL-6
Lane 1 - *A.oryzae* 1560-6, recipient strain
Lane 2 - Transformant T1560-AMDgla2
Lane 3 - Transformant T1560-AMDgla5
Lane 4 - Transformant T1560-AMDgla8
Lane 5 - Transformant T1560-AMDgpd1
Lane 6 - Transformant T1560-AMDgpd4
Lane 7 - Transformant T1560-AMDgpd18

A**B**

anti-*A.niger* GLAA pAbs failed to detect both hIL-6 or *A.niger* GLAA recombinant proteins (Lucena, unpublished).

Apparently, high concentration of caesium chloride added to the selective medium slowed down the growth of 1560-6 transformants. Furthermore, all transformants selected by colony size and sporulation do not seem to have been transformed by any of the hIL-6 cDNA sequence-containing plasmids. If these selective aspects depended upon the copy number of the integrated *amdS* gene being able to overcome caesium chloride action, and colonies transformed only with selective plasmid had more copies of the *amdS* gene integrated than colonies co-transformed with one of hIL-6-encoding plasmid, then this possibly explains the difficulties in screening 1560-6 transformants, in particular, under this conditions. Another approach would have been to use a defective *amdS* strain as the host.

1.2 Improvement of hIL-6 production

A strategy of mutagenesis and screening can be used in attempting to increase the yield of heterologous protein secreted by filamentous fungi. For example, the production of chymosin from *A.niger* var. *awamori* has been improved by subsequent U.V. mutagenesis and screening (Ward, 1990). This strategy has also been used to obtain an *A.niger* protease defective mutant (*prt*⁻) exhibiting reduced extracellular protease activity (van

den Hondel et al., 1991). However, *A. oryzae* produces copious amount of extracellular proteases (Tatsumi et al., 1989 and 1991; Cheevadhanarak et al., 1991a,b and Berka et al., 1991). Thus, the extent of U.V mutagenesis for generation of *A.oryzae* protease defective mutant has to be enough to mutate as much extracellular protease-encoding genes as possible without affecting the growth and expression of the foreign gene. Such strategy could be laborious and time consuming. Therefore, we decided to improve hIL-6 production by generating *A.oryzae* 2-deoxy-D-glucose resistant mutants.

1.2.1 Isolation of 2-deoxy-D-glucose resistant mutants

Mutations selected by resistance to toxic sugars such as 2-deoxy-D-glucose and L-sorbose relieve carbon catabolite repression (Bailey & Arst, 1975). Therefore, isolation of 2-deoxy-D-glucose resistant mutants was carried out to avoid glucose repression of glucoamylase regulatory sequences in heterologous expression system.

Mutation in at least three genes leads to relief of carbon catabolite repression. The *Aspergillus creA* gene is a negative acting regulatory gene involved in the regulation of the synthesis of many carbon catabolite repressible enzymes and permeases (Bailey & Arst, 1975; Kelly, 1993; Hintz & Lagosky, 1993). The *Aspergillus creB* and *creC* are thought to encode either a major membrane protein or the enzymes involved in the

synthesis of a major membrane component. Therefore, the carbon catabolite derepression observed on *creB⁻* or *creC⁻* mutants is probably due to altered membrane functions which reduce the uptake of carbon catabolite repressing sugars (Arst, 1980; Kelly, 1993).

Transformant T1560-*glal5* was grown on CM for 5 days at 30°C. Spores were harvested by scraping the surface of the plate with saline-TWEEN 80 solution. 4×10^5 viable spores were spread onto mutagenic medium (MM, containing 1 % xylose as the carbon source, 10 mM ammonium as sole nitrogen source and 2-deoxy-D-glucose (Sigma) at the concentration of 30, 60 or 120 mM) and incubated at 30°C for 5 days. Approximately, 20 mutants were obtained on each plate which suggested that there was not a relationship between the dose and toxic effect of 2-deoxy-D-glucose or that it had reached a plateau at the dose of 30 mM under these conditions. The same protocol was followed using transformant T1560-*gpd1*, although no mutants were obtained.

1.2.2 Screening of 2-deoxy-D-glucose mutants

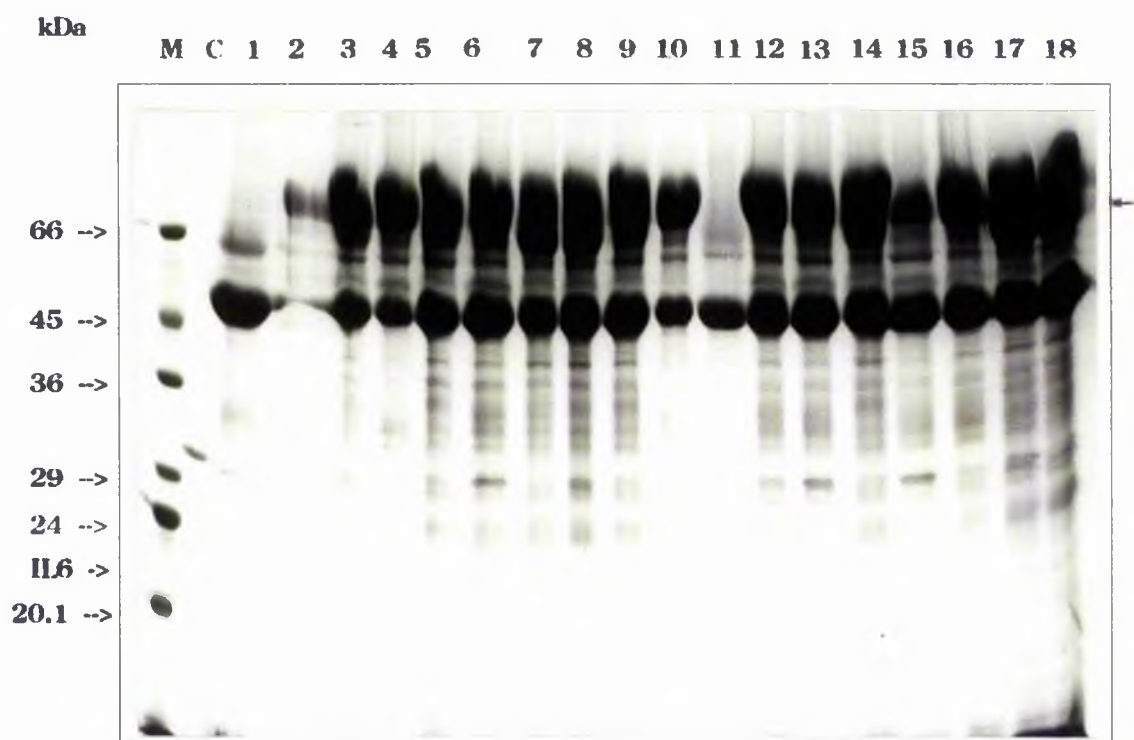
Fifteen mutants isolated by growth on the 60 mM 2-Deoxy-D-glucose plate were selected. 10^8 spores from each mutant were inoculated into 100 ml 1 % maltose MM containing ammonium as the sole nitrogen source, and incubated with agitation at 30°C for 48 h. Culture filtrates were harvested and aliquots of 1 ml were

Figure 3.1.20 Screening of 2-deoxy-D-glucose resistant mutants from transformant T1560-gla15. A SDS-polyacrylamide gel electrophoresis of proteins contained in 1 ml of culture filtrates from mutants grown in MM containing 1 % maltose as the sole carbon source is shown in Panel A. The arrow on the right shows the *A.niger* glucoamylase band position. A western blot of these proteins probed with anti-hIL6 pAb and anti-rabbit IgG conjugated to alkaline phosphatase is shown in Panel B.

Lane M - Molecular weight markers
Lane C - *S.cerevisiae* recombinant hIL-6 marker
Lane 1 - *A.oryzae* 1560-6, recipient strain
Lane 2 - Transformant T1560-gpd1, as a positive marker
Lane 3 - Transformant T1560-gla15
Lane 4 - Mutant Mgl15-1
Lane 5 - Mutant Mgl15-2
Lane 6 - Mutant Mgl15-3
Lane 7 - Mutant Mgl15-4
Lane 8 - Mutant Mgl15-5
Lane 9 - Mutant Mgl15-6
Lane 10 - Mutant Mgl15-7
Lane 11 - Mutant Mgl15-8
Lane 12 - Mutant Mgl15-9
Lane 13 - Mutant Mgl15-10
Lane 14 - Mutant Mgl15-11
Lane 15 - Mutant Mgl15-12
Lane 16 - Mutant Mgl15-13
Lane 17 - Mutant Mgl15-14
Lane 18 - Mutant Mgl15-15

freeze-dried overnight. The resulting powder was used in SDS-PAGE analysis and western blotting (Figure 3.1.20). Transformant T1560-gpd1 was used as positive control. Transformant T1560-gla15 and almost all mutants obtained from T1560-gla15 strain showed in a Coomassie stained gel a large band with a relative mobility of approximately 100-120 kDa except for mutant Mglal5-8. The western blot probed with anti-hIL6 pAb, revealed two set of bands. The one migrates with the same relative mobility as the glucoamylase protein and suggests to be a fused GLAA/hIL6 protein. In transformant T1560-gpd1 control only a weak 100 kDa band was found which was not seen on the recipient strain 1560-6. While in the mutants these high molecular weight bands were shown to range from 60 kDa to 125 kDa which might represent the protease degradation and/or different glycosylation patterns of the fused GLAA/hIL-6 protein. The mutant Mglal5-8 does not seem to secrete a fused GLAA/hIL6 protein. The other set of bands migrates with relative mobility ranging from 14 kDa to 25 kDa. The two largest bands in T1560-gpd1 control have the apparent molecular weight of 22 kDa and 25 kDa as judged by the western blot showed in Figure 3.1.17. The majority of the Mglal5 mutants showed the same larger band as detected in transformant T1560-gpd1 and one or more additional smaller bands. It is more likely that the 25 kDa protein band represents the mature form of the recombinant *A.oryzae* hIL-6 protein while the smaller bands are the product of the proteolytic degradation of this 25 kDa

A



B

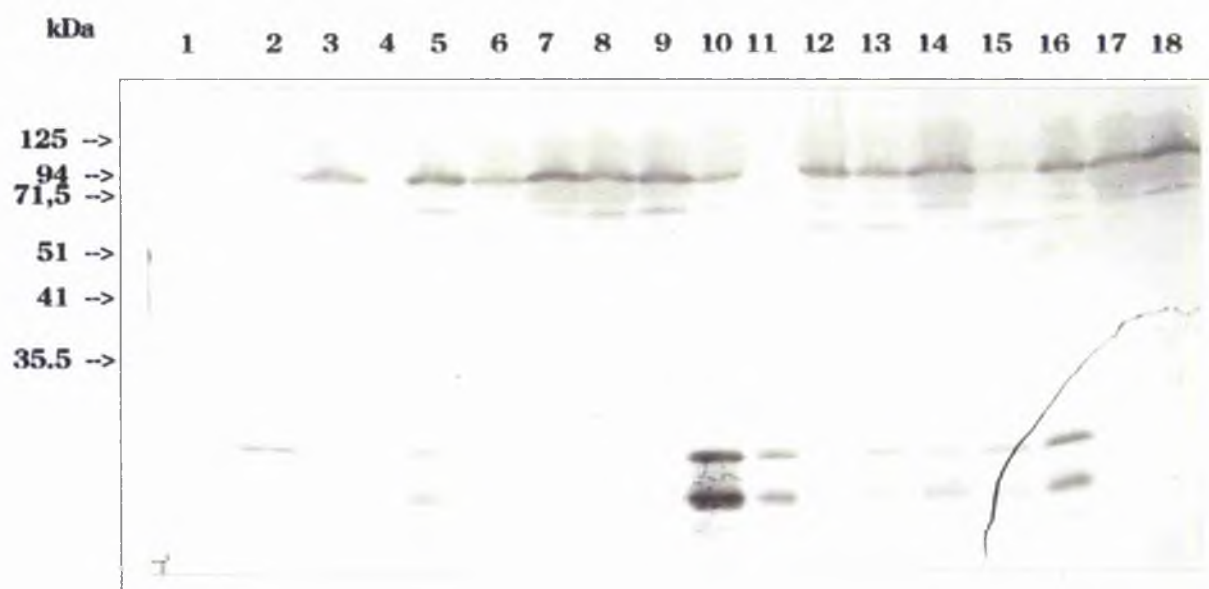


Figure 3.1.21 Southern Hybridization studies. *Hind*III digested genomic DNA from *A.oryzae* 1560-6 recipient strain (5 μ g), transformant T1560-gpd1 (5 μ g) and T1560-gla15 (2 μ g) and mutant Mgl15-7 (5 μ g) and also, different concentration of a hIL6 cDNA fragment were loaded on a 0.8 % agarose gel. The loaded hIL6 fragments were used to determine the number of copies of hIL6 integrated in 5 μ g of genomic DNA. Electrophoresis was carried out at 50 V. The DNA was Southern blotted onto a nylon membrane and cross-linked by U.V. light exposure. The filter was hybridized with a specific hIL-6 probe under stringent conditions.

- Lane 1 - 5 μ g genomic DNA of *A.oryzae* 1560-6 strain
- Lane 2 - 2 μ g genomic DNA of transformant T1560-gla15
- Lane 3 - 5 μ g genomic DNA of mutant Mgl15-7
- Lane 4 - 5 μ g genomic DNA of transformant T1560-gpd1
- Lane 5 - 1 copy equivalent hIL-6 gene
- Lane 6 - 5 copies equivalent hIL-6 gene
- Lane 7 - 10 copies equivalent hIL-6 gene
- Lane 8 - 50 copies equivalent hIL-6 gene
- Lane 9 - 100 copies equivalent hIL-6 gene

protein band. Apparently, the mutant Mgl15-7 showed substantial improvement in hIL-6 production compared to transformants T1560-gpd1 and T1560-gla15 and it is important to notice that the number of expression cassette copies integrated in 5 μ g genomic DNA of mutant Mgl15-7 was shown to be, at least, five-fold less than in transformant T1560-gpd1 (Figure 3.1.21).

One interesting observation on the western blot probed with anti-hIL6 pAB was that mutants Mgl15-7 and -8 appeared to have different properties. The mutant Mgl15-8 did not seem to secrete hIL-6 as a fused protein. 2-deoxy-D-glucose resistance mutants have been shown to affect the synthesis of certain enzymes much more strongly than others (Bailey & Arst, 1975). For instance, *Neurospora crassa* 2-deoxy-D-glucose resistant mutants grown in glucose medium showed differences on glucoamylase activity (Allen et al., 1989). Therefore, the different hIL-6 secretion behaviour in Mgl15-7 and Mgl15-8 could be due to the different 2-deoxy-D-glucose toxic effects in the mutant glucose-repressible enzymes.

1.2.3 Time course of hIL-6 production

In order to determine the hIL-6 secretion and extracellular degradation profile a time course of hIL-6 production by transformants T1560-gpd1 and T1560-gla15 and mutants Mgl15-7 and Mgl15-8 were carried out as follows.

1 2 3 4 5 6 7 8 9

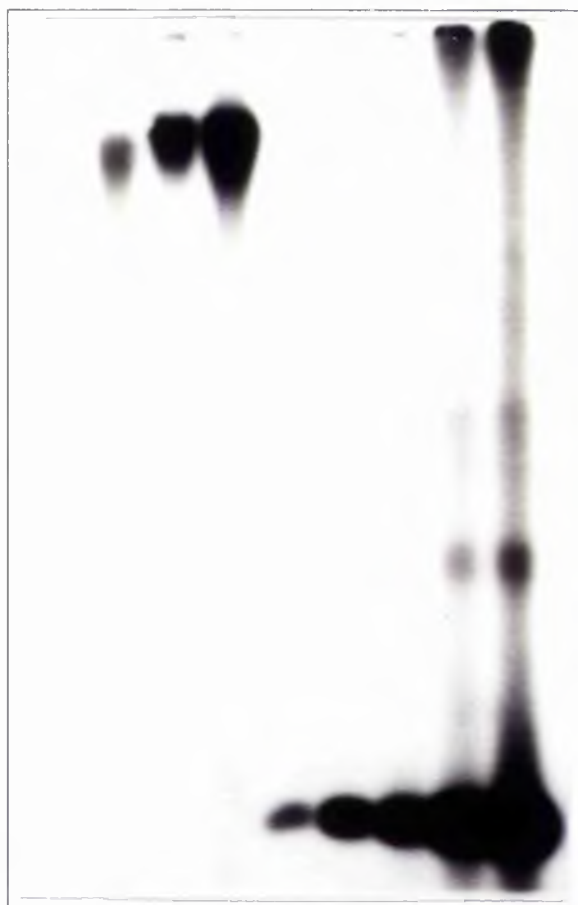


Figure 3.1.22 Studies of 2-deoxy-D-glucose resistant mutants Mgl15-7 and Mgl15-8. *A.oryzae* strains 1560-6, T1560-gla15, T1560-gpd1, Mgl15-7 and Mgl15-8 were grown in MM containing 1 % maltose as the sole carbon source. Proteins contained in 1 ml of culture filtrates from these strains were separated by a SDS-PAGE. The proteins were visualized by Coomassie blue staining. The *A.niger* glucoamylase band is indicated on the right (Panel A) or on the left (Panel B)

Panel A -

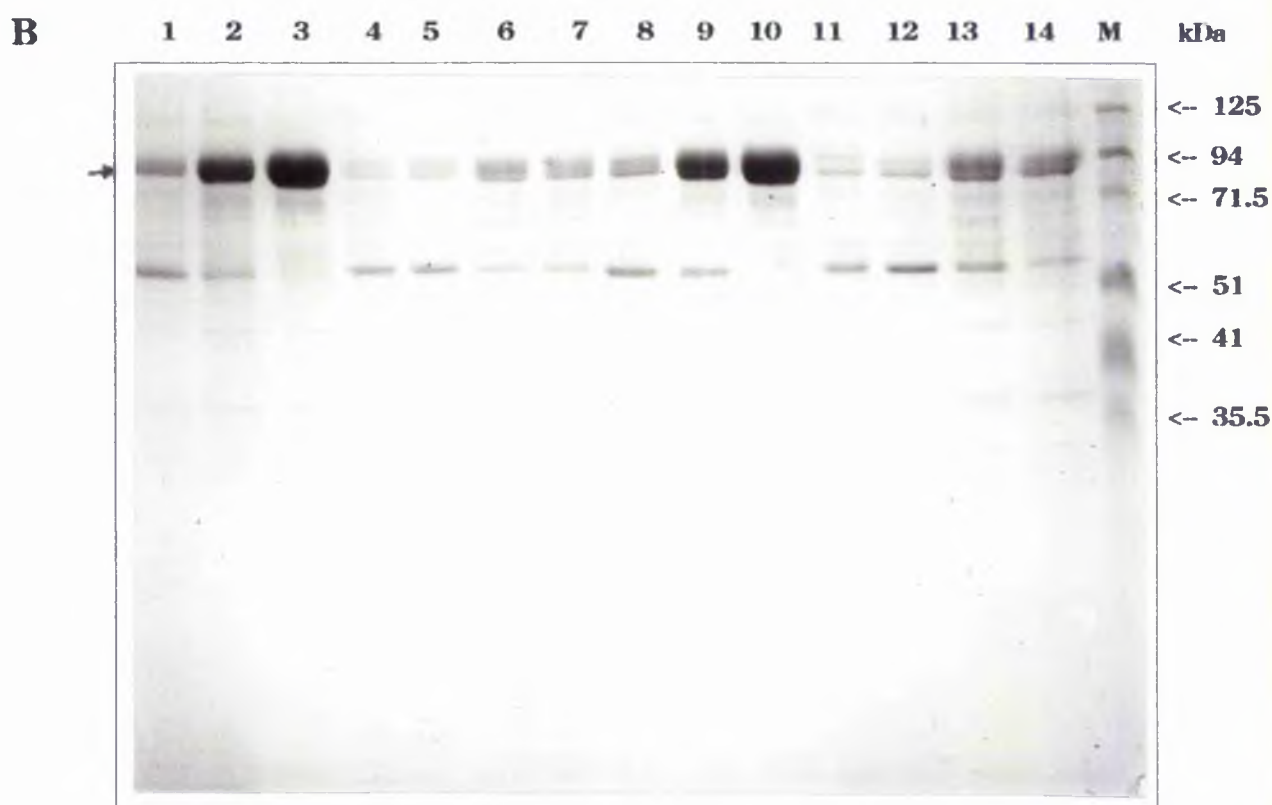
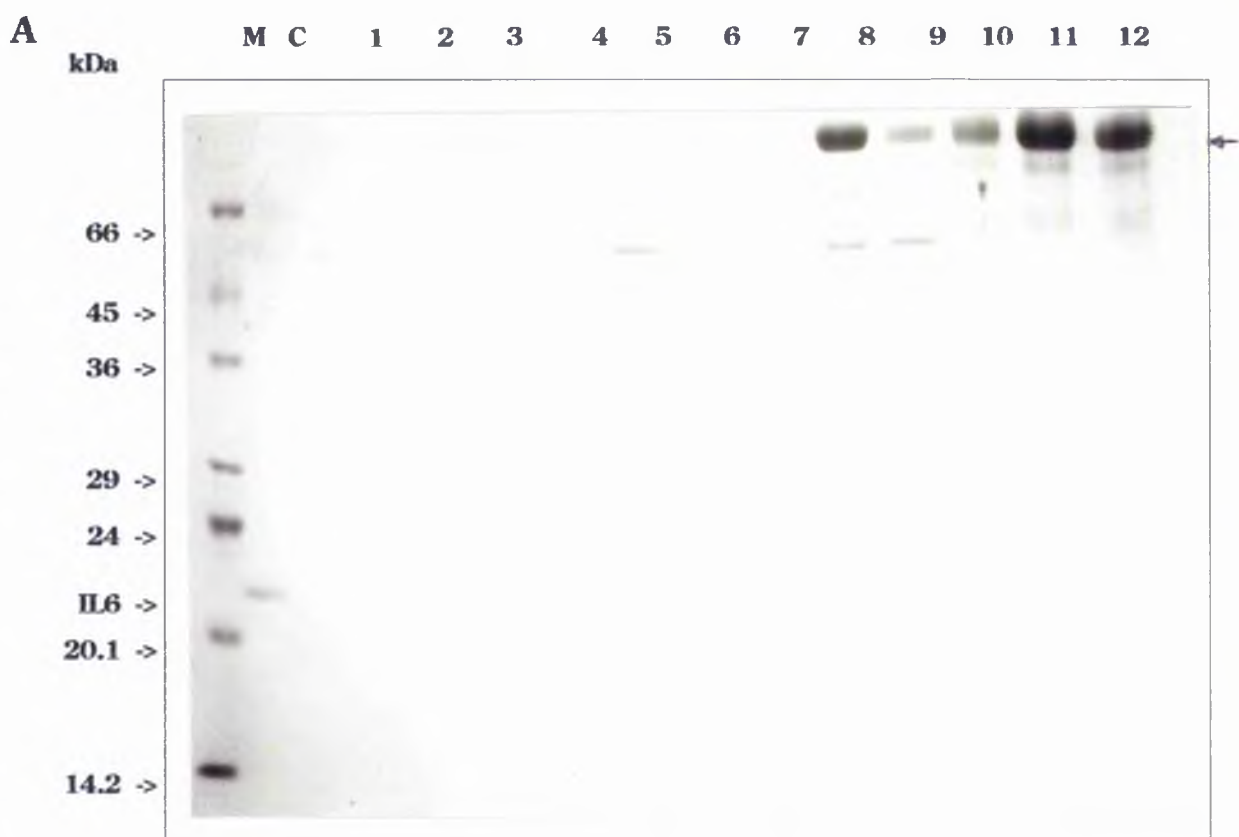
- Lane M - Molecular weight markers
- Lane C - *S.cerevisiae* recombinant hIL-6
- Lanes 1 to 4 - *A.oryzae* 1560-6, recipient strain
Time course of 12,24,36 and 48 h
- Lanes 5 to 8 - Transformant T1560-gpd1, positive marker
Time course of 12,24,36 and 48 h
- Lanes 9 to 12 - Transformant T1560-gla15
Time course of 12,24,36 and 48 h

Panel B -

- Lane M - Molecular weight markers
- Lanes 1 to 3 - Mutant Mgl15-7
Time course of 12,24 and 36 h
- Lanes 4 to 7 - Mutant Mgl15-7 grown in presence of 2-deoxy-D-glucose.
Time course of 12,24,36 and 48 h
- Lanes 8 to 10 - Mutant Mgl15-8
Time course of 12,24 and 36 h
- Lanes 11 to 14 - Mutant Mgl15-8 grown in presence of 2-deoxy-D-glucose.
Time course of 12,24 and 36 h

10^7 spores from recipient strain 1560-6, transformants T1560-gpd1 and T1560-gla15 and mutants Mgl15-7 and Mgl15-8 were harvested from a 5 day-old plate with saline-TWEEN 80 and were inoculated in 10 ml of 1 % maltose MM, containing 10 mM ammonium as the sole nitrogen source, in four vials per strain. Arginine was supplied to the 1560-6 novo strain. Mutants Mgl15-7 and Mgl15-8 were also each inoculated as above in 10 ml of MM supplemented with 60 mM of 2-deoxy-D-glucose. All cultures were incubated at 30°C with agitation. After 12, 24, 36 and 48 h of incubation one vial tube of each batch was removed. Culture filtrates were lyophilized overnight in aliquots of 1 ml and the resulting powder was used in SDS-PAGE analysis and western blotting.

The results of the SDS-PAGE analysis revealed (Figure 3.1.22) the higher level of glucoamylase secretion in transformant T1560-gpd1 was achieved after 48 h of growth, whereas in transformant T1560-gla15 and mutants Mgl15-7 and Mgl15-8 was after 36 h. This could be related to the maltose induction on the *glaA* promoter. The addition of 60 mM 2-deoxy-D-glucose in the medium does not affect the biomass production of mutants Mgl15-7 and Mgl15-8 (Lucena, unpublished). However, the production of glucoamylase seemed to be reduced. This finding indicated that this glucose analogue might have had negative effects on permeases or other enzymes which affected the induction of glucoamylase synthesis



by maltose or increased its intracellular degradation or blocked its secretion.

A western blot probed with anti-*A.niger* GLAA pAb (Figure 3.1.23) confirmed that the higher level of glucoamylase secretion in strain T1560-gpd1 was at 48 h of incubation. In contrast, the level of glucoamylase secretion in the strains T1560-gla15, Mgl15-7 and Mgl15-8 achieved high levels at 24 h and were still high at 48 h. However, the degradation level of glucoamylase seemed to increase with time. With regard to Mgl15-7 and Mgl15-8 strains grown in the presence of this glucose analogue, the amount of glucoamylase secreted seemed to increase slightly with the time, and the extracellular protease activity did not appear to be strong enough to form a wide smeared band.

A western blot probed with anti-hIL6 pAb (Figure 3.1.24) revealed that all strains analysed except the recipient strain secreted into the medium a high molecular weight protein band of approximately 120 kDa which suggested to be the fused glucoamylase/hIL6 protein. Moreover, the strain T1560-gpd1 showed at least four more bands, the two larger bands with relative molecular weight of approximately 20-25 kDa, the other two smaller bands are less dense. Approximately the same pattern was detected in strains T1560-gla15, Mgl15-7 and Mgl15-8, mainly, at 24 h. The presence of free hIL-6 protein in strains Tgla15-7 and Tgla15-8 grown in the presence of 2-deoxy-D-glucose was not significant.

Figure 3.1.23 Studies of 2-deoxy-D-glucose resistant mutants Mgl15-7 and Mgl15-8. Proteins contained in 1 ml of culture filtrates from *A.oryzae* strains 1560-6, T1560-gla15, T1560-gpd1, Mgl15-7 and Mgl15-8 grown in MM containing 1 % maltose as sole carbon source were western blotted onto nitrocellulose membrane and probed with anti-*A.niger* GLAA pAb and anti-rabbit IgG conjugated to alkaline phosphatase.

Panel A -

Lane M - Molecular weight markers

Lanes 1 to 4 - *A.oryzae* 1560-6, recipient strain
Time course of 12,24,36 and 48 h

Lanes 5 to 8 - Transformant T1560-gpd1, positive marker
Time course of 12,24,36 and 48 h

Lanes 9 to 12 - Transformant T1560-gla15
Time course of 12,24,36 and 48 h

Panel B -

Lane M - Molecular weight markers

Lanes 1 to 3 - Mutant Mgl15-7
Time course of 12,24 and 36 h

Lanes 4 to 7 - Mutant Mgl15-7 grown in presence of
2-deoxy-D-glucose.
Time course of 12,24,36 and 48 h

Lanes 8 to 10 - Mutant Mgl15-8
Time course of 12,24 and 36 h

Lanes 11 to 14 - Mutant Mgl15-8 grown in presence of
2-deoxy-D-glucose.
Time course of 12,24 and 36 h

A



B

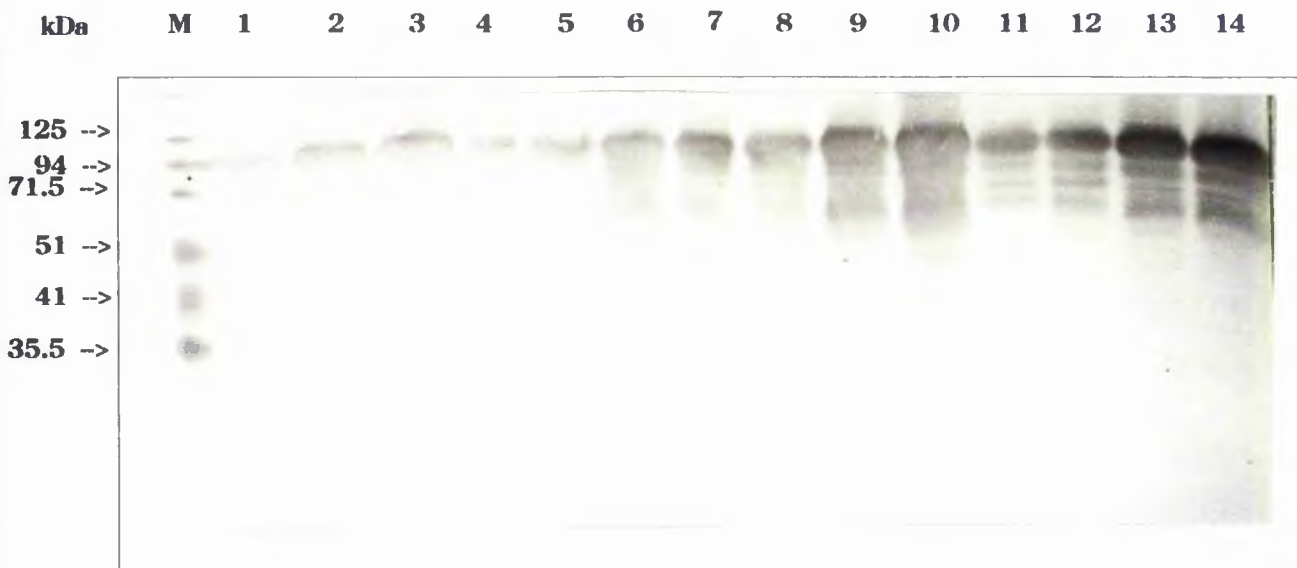


Figure 3.1.24 Studies of 2-deoxy-D-glucose resistant mutants Mgl15-7 and Mgl15-8. Proteins contained in 1 ml of culture filtrates from *A.oryzae* strains 1560-6, T1560-gla15, T1560-gpd1, Mgl15-7 and Mgl15-8 grown in MM containing 1 % maltose as sole carbon source were western blotted onto nitrocellulose membrane and probed with anti-hIL6 pAb and anti-rabbit IgG conjugated to alkaline phosphatase.

Panel A -

Lane M - Molecular weight markers

Lanes 1 to 4 - *A.oryzae* 1560-6, recipient strain

Time course of 12,24,36 and 48 h

Lanes 5 to 8 - Transformant T1560-gpd1, positive marker

Time course of 12,24,36 and 48 h

Lanes 9 to 12 - Transformant T1560-gla15

Time course of 12,24,36 and 48 h

Panel B -

Lane M - Molecular weight markers

Lanes 1 to 3 - Mutant Mgl15-7

Time course of 12,24 and 36 h

Lanes 4 to 7 - Mutant Mgl15-7 grown in presence of 2-deoxy-D-glucose.

Time course of 12,24,36 and 48 h

Lanes 8 to 10 - Mutant Mgl15-8

Time course of 12,24 and 36 h

Lanes 11 to 14 - Mutant Mgl15-8 grown in presence of 2-deoxy-D-glucose.

Time course of 12,24 and 36 h

A

kDa **M** **1** **2** **3** **4** **5** **6** **7** **8** **9** **10** **11** **12**

125 ->
94 ->
71.5 ->
51 ->
41 ->
35.5 ->



B

kDa **1** **2** **3** **4** **5** **6** **7** **8** **9** **10** **11** **12** **13** **14**

125 ->
94 ->
71.5 ->
51 ->
41 ->
35.5 ->



The free form of hIL-6 molecules were secreted into the growth medium by transformants T1560-gla15 and its 2-deoxy-D-glucose resistant mutants, apparently, in higher levels than that secreted by transformant T1560-gpd1. However, there was no apparent differences in the level of hIL-6 secreted by T1560-gla15 or its 2-deoxy-D-glucose mutants Mgl15-7 and Mgl15-8. Nevertheless, the following considerations were possible: (1) The time of detection of hIL-6 into the medium varied with producer strain. (2) In maltose medium the inducible *glaA* promoter increased not only the amount of glucoamylase/hIL6 protein synthesized but also yielded earlier secretion. (3) On the basis of comparison of the glucoamylase and hIL-6 bands revealed on the western blot, the extracellular protease activity that took place after 24 h of growth might have been strong as judged by the smeared glucoamylase band. Furthermore, hIL-6 degradation seemed to be more susceptible to proteolytic degradation than glucoamylase protein. This might indicate that hIL-6 being a mammalian protein might have exposed on the surface of its molecule, amino acid sequences which were recognised and degraded by one of the *A.oryzae* extracellular proteases. The recombinant glucoamylase probably was more resistant to this proteolytic activity due to it's 67 % amino acid homology to the *A.oryzae* glucoamylase. (4) Moreover, comparing the width of the hIL6 band with the glucoamylase band at corresponding times, we can speculate that the synthesized hIL-6 protein might have

also been degraded by *A.oryzae* intracellular proteases. (5) Finally, the results suggested that glucose does not seemed to inhibit the *A.niger* glucoamylase expression in transformant T1560-gla15 (Figure 3.1.16).

1.2.4 Influence of the carbon source on Mglal5-7 growth and hIL-6 production

In order to assess the influence of the carbon source in Mglal5-7 growth and hIL-6 production the following experiment was carried out.

4×10^8 spores from a five day-old culture were incubated in nine vials containing 100 ml of MM buffered with 10 mM pH 6.5 phosphate buffer. Each of the three vials contained 1 % glucose, 1 % maltose or 1 % xylose as the sole carbon source and 10 mM of ammonium as the sole nitrogen source. Incubation was carried out with agitation at 25°C for 20 h. Low temperature was used in order to reduced biomass production. After incubation a concentrated maltose solution was added into two vials of the glucose cultures, two of maltose cultures and two of xylose cultures, all at 1 % final concentration, in order to induce hIL-6 protein synthesis. One vial of each different carbon cultures where maltose was added were incubated with agitation at 25°C for further 6 h. Another vial of each different carbon cultures which was added maltose, as well those control cultures where no maltose was added, were incubated with agitation at 30°C

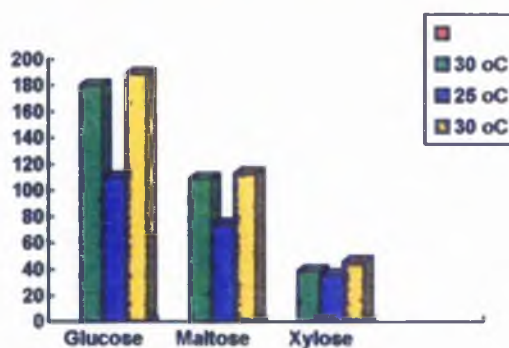
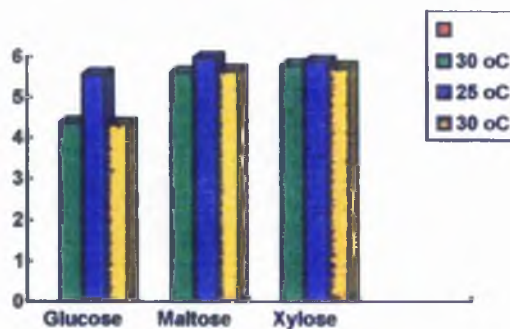
for 6 h. 1 ml aliquots of the culture filtrates were lyophilized overnight and the resulting powder was used in western blotting experiments.

The growth of the mycelia was assessed by dried biomass quantification. The pH measurement was also important as one indicative of the metabolic state of culture. The highest biomass production the lowest pH value was found. Moreover, the results showed that the pH values varied with the temperature of incubation and type of carbon source used even though the media had been buffered (Table 3.1.9).

Cultures moved from 25°C to 30°C incubation showed higher biomass production and also higher glucoamylase secretion than those cultures where maltose (an inducer for glucoamylase synthesis) were added, but left at 25°C incubation instead. The highest biomass production was observed in cultures moved to 30°C incubation where maltose was added.

The results showed in the western blot probed with anti-*A.niger* GLAA pAb (Figure 3.1.25) indicated: (1) Glucoamylase production in the xylose cultures was reduced and was not increased by increasing the incubation temperature nor significantly by maltose addition. (2) Glucoamylase production in the glucose cultures was insignificant when carried out at 25°C even though an inducer (maltose) was added. However, culture grown at 30°C in absence of maltose yielded a wide

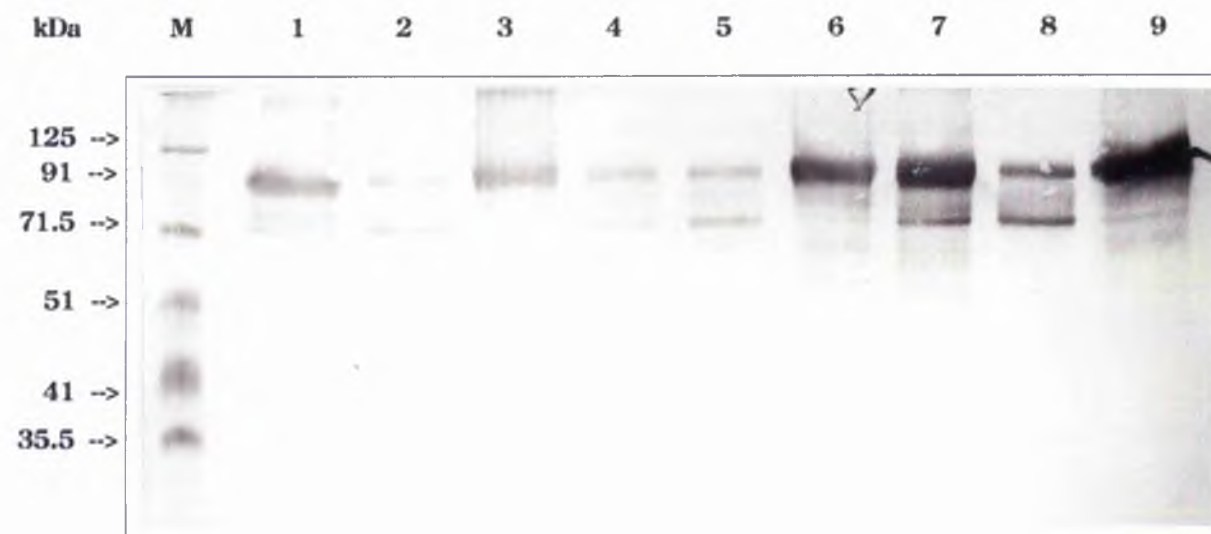
Table 3.1.9 Temperature and pH effects on hIL-6
production by mutant Mgl15-7 grown in MM containing
different carbon sources. Mutant Mgl15-7 was grown in pH
6.5 buffered glucose MM, maltose MM or xylose MM. After a
initial incubation of 20 h at 25°C, cultures were either
incubated at 30°C or left at 25°C for 6 h. The induction of
glucoamylase synthesis by maltose was assessed. The pH's of
the culture filtrates and the biomass produced are shown in
the table. The differences of the final pH's and biomass in
these cultures are shown in a graphic representation (top
and bottom respectively). Green, for culture incubated at
30°C, but no addition of maltose; blue, for culture
incubated at 25°C after maltose addition and yellow, for
culture incubated at 30°C after maltose addition.



Substrate	Temperature	Final pH	dry weight (mg)
Glucose	30 oC (not induced)	4.36	180
Glucose	25 oC (induced)	5.55	110
Glucose	30 oC (induced)	4.33	189
Maltose	30 oC (not induced)	5.60	109
Maltose	25 oC (induced)	5.96	74
Maltose	30 oC (induced)	5.65	113
Xylose	30 oC (not induced)	5.78	39
Xylose	25 oC (induced)	5.86	36
Xylose	30 oC (induced)	5.73	46

Figure 3.1.25 Temperature and pH effects on glucoamylase production by mutant Mgla15-7 grown in MM containing different carbon sources. Mutant Mgla15-7 was grown in pH 6.5 buffered glucose MM, maltose MM or xylose MM. After a initial incubation of 20 h at 25°C, cultures were either incubated at 30°C or left at 25°C for 6 h. The induction of glucoamylase synthesis by maltose was assessed. Proteins of 1 ml of culture filtrates were western blotted onto nitrocellulose membrane and probed with anti-*A.niger* GLAA pAB and anti- rabbit IgG conjugated to alkaline phosphatase.

- Lane 1 - Molecular weight markers
- Lane 2 - Mgla15-7 grown in glucose MM at 30°C
- Lane 3 - Mgla15-7 grown in glucose MM at 25°C, induced
- Lane 4 - Mgla15-7 grown in glucose MM at 30°C, induced
- Lane 5 - Mgla15-7 grown in xylose MM at 30°C
- Lane 6 - Mgla15-7 grown in xylose MM at 25°C, induced
- Lane 7 - Mgla15-7 grown in xylose MM at 30°C, induced
- Lane 8 - Mgla15-7 grown in maltose MM at 30°C
- Lane 9 - Mgla15-7 grown in maltose MM at 25°C, induced
- Lane 10 - Mgla15-7 grown in maltose MM at 30°C, induced



glucoamylase band which was enlarged by maltose induction. (3) Glucoamylase production in the maltose cultures was high and induced by addition of concentrated maltose. This finding showed the feasibility to produce glucoamylase in high levels by fed-batch culture. (4) Finally, Mgl15-7 mutant does not seem to utilize xylose as carbon source as well as utilizes glucose and maltose, which could be due changes in membrane permeability.

Surprisingly, no free or fused hIL-6 protein corresponding bands were detected on western blot probed with anti-hIL6 pAb and anti-rabbit IgG conjugated to alkaline phosphatase, though the yeast recombinant hIL-6 marker was detected (Lucena, unpublished). One possibility is if these growth conditions facilitated qualitative changes in the recombinant hIL-6 thermostability causing its premature degradation (Allen et al., 1989).

1.2.5 Fermentation process for hIL-6 production

The viability to produce hIL-6 in solid state culture was assessed. The protocol followed was detailed in Table 3.1.10. 10^7 spores of Mgl15-7 strain were inoculated on 10 g wheat bran containing 40 % or 80 % saline (w/v) and on 10 g rice. 10^7 spores were also inoculated in 1 % maltose liquid MM as control. In addition, mycelium grown either in liquid or on solid

Table 3.1.10 Protocol for hIL-6 production by *Aspergillus oryzae*. Mutant Mgl15-7 was grown either in liquid culture or solid state culture as follows (see text for details).

Parameters	culture one	culture two	culture three	culture four	culture five	culture six	culture seven	culture eight	culture nine
Media composition									
1. 2 % maltose MM buffered to pH 6.5	yes	yes	no	yes	no	no	no	no	no
2. Solid media									
10 g Rice	no	no	yes	no	no	no	yes	yes	yes
5 g Wheat bran	no	no	no	no	yes	yes	no	no	no
Growth parameters									
Inoculum age (days)	5	5	5	5	5	5	5	1	1
Inoculum size (spores)	10 ⁷	10 ⁷	10 ⁷	10 ⁷	10 ⁷	10 ⁷	10 ⁷	no	no
(mycelia from culture)	no	no	no	no	no	no	no	4	3
Temperature	30°C	30°C	28°C	30°C	28°C	28°C	28°C	28°C	28°C
Humidity (%)	NA	NA	20	NA	40	80	20	20	20
Duration (h)	26	26	26	24	24	24	24	24	24
Induction									
Inductor (maltose)	no	yes	yes	no	no	no	no	no	no
Duration (h)	NA	6	6	NA	NA	NA	NA	NA	NA

NA not applicable

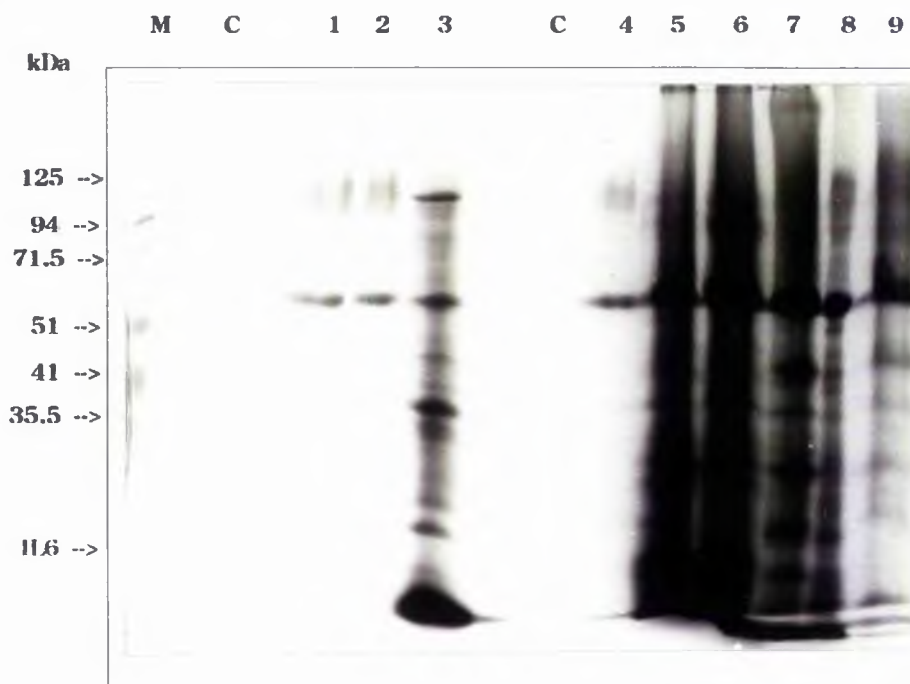
state cultures were inoculated on 10 g rice in order to examine the optimum type of inoculum. Incubation was carried out in 28°C incubator or in 30°C shaker for 24 h. The solid state cultures were washed with 50 ml sterile distilled water, by shaking for 20 min, at room temperature. 1 ml aliquots of these culture washes and of the liquid culture filtrate were lyophilized overnight. The resulting powder was used in SDS-PAGE analysis and western blotting.

The results showed high production of *A.niger* glucoamylase in solid state fermentation, concomitant with low detection of fused glucoamylase/hIL-6 and no free form of the hIL-6 protein was detected (Figure 3.1.26, Lanes 4 to 9). The failure in detected free form of hIL-6 molecules in the culture washes might be related to the concomitant production of *A.oryzae* extracellular proteases by this surface cultivation (Ramamurthy & Kothari, 1993). The high level of detection of *A.niger* glucoamylase might be due to the 67 % of nucleotide homology between *A.niger* and *A.oryzae* glucoamylase, which protected it against proteolytic degradation by *A.oryzae* extracellular proteases. Moreover, the fusion GLAA/hIL6 protein appeared to protect hIL-6 degradation by masking the protease recognizing sites on hIL-6 molecule or maybe, this fused GLAA/hIL6 protein was still detected as a result of protease saturation.

Figure 3.1.26 Analysis of hIL-6 production by mutant Mgl15-7. Mutant Mgl15-7 was grown in different conditions as shown in Table 1.10. Proteins of 1 ml of culture filtrates were separated by SDS-polyacrylamide gel electrophoresis. Gels were either stained with Coomassie blue and destained with methanol - acetic acid solution (Panel A), or western blotted. Filters were probed either with anti-*A.niger* GLAA (Panel B) or anti-hIL6 (Panel C) pAbs. Both filters were then probed with protein A conjugated to peroxidase.

- Lane M - Molecular weight markers
- Lane C - *S.cerevisiae* recombinant hIL-6
- Lane 1 - Mgl15-7 grown in maltose MM
- Lane 2 - Mgl15-7 grown in maltose MM, induction
- Lane 3 - Mgl15-7 grown on rice, induction
- Lane 4 - Mgl15-7 grown in maltose MM
- Lane 5 - Mgl15-7 grown on wheat bran (40 % humidity)
- Lane 6 - Mgl15-7 grown on wheat bran (80 % humidity)
- Lane 7 - Mgl15-7 grown on rice (inoculum = spores)
- Lane 8 - Mgl15-7 grown on rice (inoculum = mycelium)
- Lane 9 - Mgl15-7 grown on rice (inoculum = mycelium)

A



B

M 1 2 3 4 5 6 7 8 9



C

kDa M C 1 2 3 C 4 5 6 7 8 9



In parallel other experiment was carried out in order to examine the possibility to improve the hIL-6 production by fed-batch fermentation.

10^7 spores from five-day old culture of Mgl15-7 strain were inoculated in 50 ml of 10 mM phosphate buffered MM pH 6.5 containing 1 % maltose as the sole carbon source and 10 mM ammonium as the sole nitrogen source, in duplicate. The incubation was carried out with agitation, at 30°C for 26 h. 10 g rice solid medium was also inoculated with 10^7 spores contained in 2 ml of saline. This solid state culture was placed in 28°C incubator for 26 h. Maltose was added at final concentration of 2 % to the solid and one of the liquid cultures 5 h before harvesting. The recombinant hIL-6 protein was detected in 1 ml aliquots of culture filtrates and culture washes by the chemoluminescence method (ECL reagents, Amersham) within 10 min film exposure .

Western blot analysis showed that the free form of hIL-6 protein was produced in buffered 1 % maltose MM at low level, but higher level was achieved by adding maltose either to the liquid or solid state cultures (Figure 3.1.26, Lanes 1, 2 and 3). Approximately, 5 μ g of yeast recombinant hIL-6 was used as a control. The majority of secreted hIL-6 was found in a free form, only small amount was found fused to glucoamylase. Nevertheless, the production levels of the recombinant glucoamylase were higher than that obtained for hIL-6

suggesting the selective proteolytic degradation of hIL-6 protein.

SECTION II :

CONSTRUCTION OF AN α -AMYLASE EXPRESSION VECTOR

Due to our interest to express high levels of correctly processed heterologous proteins using *Aspergillus oryzae* as a host, the expression vector outlined in Figure 3.2.1 was designed. The construct consists of the entire *A.oryzae amy3* gene (Wirsel et al., 1989), in which the stop codon was mutagenized by Polymerase chain Reaction in order to create cloning sites.

2.1 PCR Procedure

2.1.1 Design of the primers

The *A. oryzae amy3* gene was amplified using PCR technology. The primers were designed as outlined in Figure 3.2.2. The 5' primer is 41 bases long and has a melting temperature of 50°C. Four extra nucleotides were added upstream the *amy3 BglIII* site at the 5'end of the primer for facilitating restriction digestion in further cloning procedures. The 3' primer is 25 bases long and has a melting temperature of 68°C. The *amy3 SphI* site was conserved and an extra four bases were added

Figure 3.2.1 Flow chart of pSTA1204 construction. First, the 3 kb *EcoRI-SphI* fragment of pSTA900 (9 kb) which harbours the *A.oryzae amy3* gene (shown in red) was inserted into *EcoRI-SphI* site of pUC18 generating pSTA1210 (5.5 kb). Second, *HindIII* site of the pUC18 polylinker located downstream the *amy3* gene was deleted by Klenow reaction. Finally, the *amy3* stop codon was mutated in order to create a *StuI* and *HindIII* cloning sites by PCR technology. The resulting plasmid, pSTA1204, was used in further experiments.

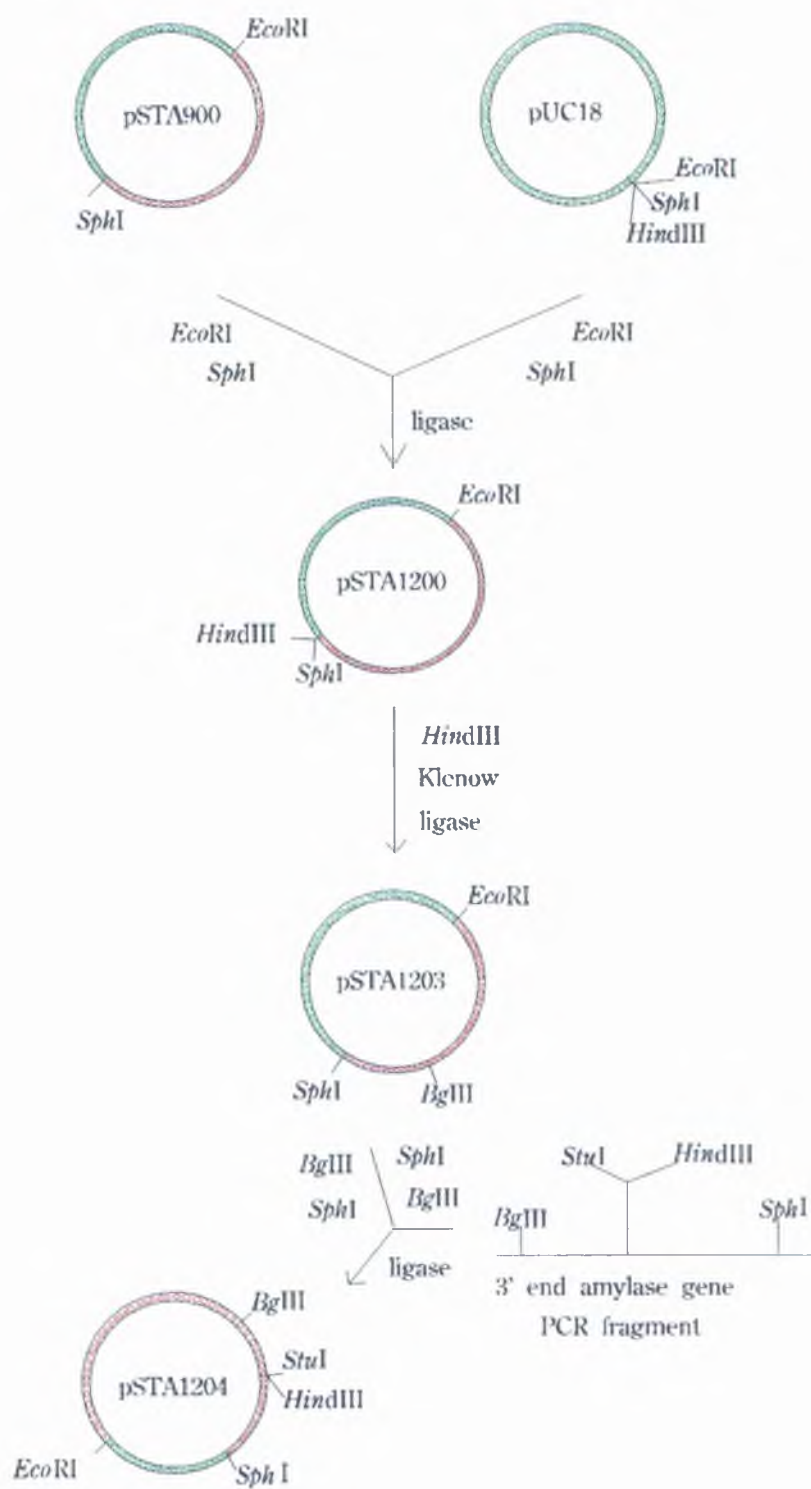


Figure 3.2.2 Design of primers. The stop codon of *A.oryzae* *amy3* gene was mutated in order to create the *StuI* and *HindIII* cloning sites and a KEX2-site. The bottom sequences in Panel A and B represent the 5' *amy3* primer and the 3' *amy3* primer respectively. The top sequences in each panel are the natural *amy3* sequences. Red line markers the stop codon. Green lines are shown either natural or created restriction endonucleases sites. Blue line underlines the KEX2-site.

A - The 5' *amy3* primer

5' _____ 3'
GC AAG ATC TGT AGT AGC TCG TGA AGG GTG GAG AGT ATA TGA TCG TAC

5' _____ 3'
GC AAG ATC TGT AGT AGG AGG CCT AGG CTT GAG AGT ATA TGA TCG TAC

B - The 3' *amy3* primer

5' _____ 3'
AAT CGC GAT ATT ATT GGC ATG CAA A

5' _____ 3'
TTA GCG CAA TAA TAA CCG TAG GTT T

downstream to aid further digestion. 490 bp *NaeI*-*EcoRV* fragment from pSTA900, which contains the stop codon of *A.oryzae amy3* gene, was used as the template. The 5' primer carried a mutation at the *amy3* stop codon generating *StuI* and *HindIII* cloning sites and a KEX2-site.

2.1.2 PCR Conditions

Strand-separation in the first cycle was performed at 94°C for 1 min. Primer-Template hybridization was carried out for 30 sec at 45°C which is 5°C below the lower melting temperature of the primers. The temperature of the reaction was then, increased in ramp until 72°C was reached, allowing hybridization to be carried out in the presence of primer extension. Primer extension time was set at 30 sec.

The subsequent 25 cycles had a stringent condition to improve the specificity of the reaction. An annealing temperature of 55°C was used to enhance the discrimination against incorrectly annealed primers.

After PCR procedure was complete, a volume of 2 % of the reaction was loaded on a 1 % agarose in 1 X TAE buffer. The electrophoresis was run at 25 V. The PCR product obtained was 291 bp long.

2.2 Insertion of the amy3 gene into pUC18

pSTA900, containing the *A.oryzae amy3* gene (Macro, 1991), was digested with *EcoRI* and *SphI*. A fragment of 1989 bp, encoding the entire *amy3* gene and the flanking untranslated regions, was isolated and purified by centrifugal filtration through glass wool (see Materials and Methods for details). pUC18 was also digested with *EcoRI* and *SphI* and the largest fragment was purified. The two purified fragments were ligated overnight at 15°C. *E.coli DH5* α competent cells were transformed with the ligation reaction. Clones were resuspended in 25 μ l of sterile distilled water and 25 μ l of 2 x cracking buffer (100 mM NaOH; 10 mM EDTA pH 8.0; 1 % SDS (v/v); 0.05 % (w/v); 10 % glycerol (v/v)) was then added. These suspensions were loaded onto 0.8 % agarose gel. Putative positive clones were screened by comparing the size of these recombinant plasmid clones with that of pUC18. Three clones, designated pSTA1200, pSTA1201 and pSTA1202, were submitted to DNA minipreparation followed by double digestions with *EcoRI* and *SphI* to recover the insert fragment. Multiple restriction digests of pSTA1200 are shown in Figure 3.2.3, Panel A.

2.3 Deletion of the HindIII site

pSTA1200 has a *HindIII* site downstream of the *amy3* gene in the pUC18 polylinker sequence. Klenow, a large fragment of DNA polymerase I with 5' - 3' polymerase

activity and 3' - 5' exonuclease activity, was used to "fill-in" the *Hind*III site of pSTA1200. The blunt ends generated by the Klenow reaction were ligated as described in Materials and Methods. Deletion of the *Hind*III site was confirmed in clone pSTA1203 by plasmid DNA minipreparation and *Hind*III digestion (Figure 3.2.3, Panel B).

2.4 Mutagenesis of pSTA1203

pSTA1203 was digested with *Bgl*II and *Sph*I. The large fragment was isolated by electrophoresis on 0.8 % SeaPlaque GTG agarose in 1 x TBE buffer. Additionally, PCR-products of the mutated *amy3* gene were digested with *Bgl*II and *Sph*I. A 290 bp fragment was isolated by electrophoresis through 3 % NuSieve GTG agarose in 1 x TBE buffer. Both fragments were excised from the gel and placed into two different pre-weighed Eppendorf tubes. The concentrations of DNA in the gel slices were calculated, assuming 100 % recovery, by dividing the amount of DNA loaded by the volume of the gel slice.

The "in gel-ligation" reaction was carried out in a volume 3 x greater than the volume of remelted agarose used. To the reaction mixture was added 100 ng of pSTA1203 fragment and 10 ng of PCR-product. For further details regarding "in gel-ligation" procedure and *E.coli* competent cell transformation see Materials and Methods Section 2.5.11.2. The putative recombinant clones were

Figure 3.2.3 Restriction endonuclease digestion of clones obtained during the construction of α -amylase expression vector. The clones were submitted to plasmid DNA extraction followed by single or double restriction endonuclease digestion of 1-5 μ g of DNA. The DNA fragments were separated in a 1 % agarose gel.

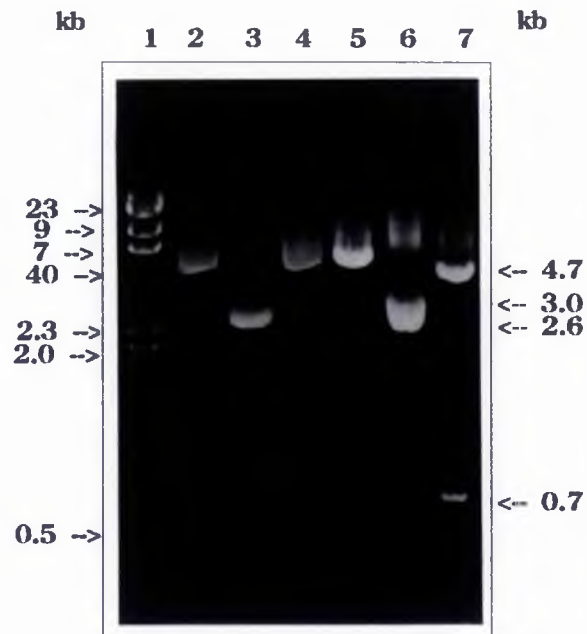
Panel A - The *A.oryzae amy3* gene was inserted into pUC18 cloning sites originating pSTA1200.

- Lane 1 - DNA size marker (*Hind*III digested lambda DNA).
- Lane 2 - pUC18 undigestion
- Lane 3 - pUC18 *Hind*III digestion
- Lane 4 - pSTA1200 undigestion
- Lane 5 - pSTA1200 *Hind*III digestion
- Lane 6 - pSTA1200 *Hind*III - *Eco*RI double digestion
- Lane 7 - pSTA1200 *Pst*I digestion

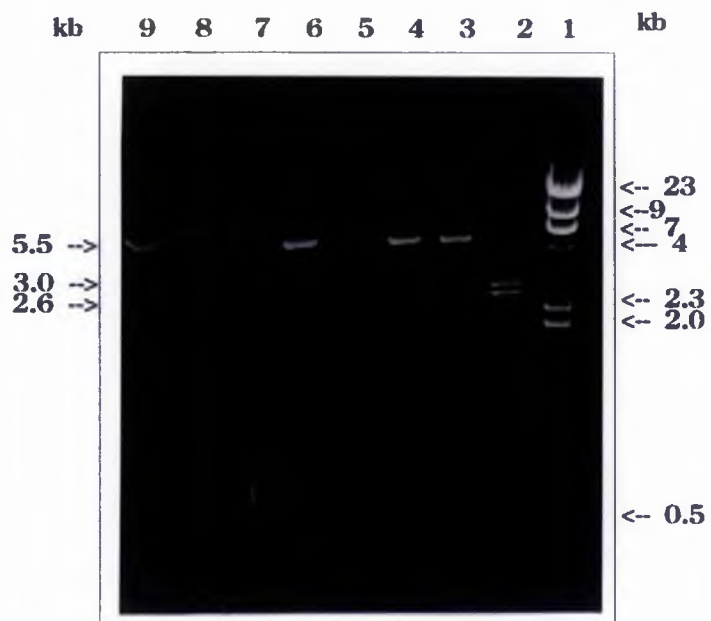
Panel B - pSTA1200 had the *Hind*III site deleted in pSTA1203. The late had sequences at the stop codon of *amy3* gene mutated to create *Stu*I and *Hind*III cloning sites in pSTA1204.

- Lane 9 - pSTA1203 undigestion
- Lane 8 - pSTA1203 *Hind*III digestion
- Lane 7 - pSTA1203 *Stu*I digestion
- Lane 6 - pSTA1203 *Eco*RI digestion
- Lane 5 - pSTA1204 undigested
- Lane 4 - pSTA1204 *Hind*III digestion
- Lane 3 - pSTA1204 *Stu*I digestion
- Lane 2 - pSTA1204 *Hind*III - *Eco*RI double digestion.
- Lane 1 - DNA size marker (*Hind*III digested lambda DNA)

A



B



submitted to DNA minipreparation and digested with *Stu*I or/and *Hind*III. The result is shown in Figure 3.2.3, Panel B.

2.5 Insertion of a Polylinker into pSTA1204

Digestion of *Stu*I and *Hind*III cloning sites on pSTA1204 might be difficult due to their close proximity. In order to facilitate the effective digestion of these cloning sites and offer more options of sites, a polylinker DNA sequence encoding the *Bss*HI, *Xba*I, *Bam*HI, *Taq*I, *Not*I and *Eco*RV sites was synthesized.

pSTA1204 was digested with *Hind*III and dephosphorylated. A 3 kb *Hind*III-fragment DNA was inserted into the *Hind*III site of the amylase vector distancing the *Stu*I site from the *Hind*III site. A positive clone was digested first with *Stu*I then with *Hind*III in order to achieve complete double digestion. The fragments were dephosphorylated and the vector fragment was isolated by electrophoresis on 0.8 % SeaPlaque agarose in 1 x TBE buffer. Both strands of synthesized DNA polylinker sequence were mixed, heated and cooled slowly to allow them to hybridize to each other. Polylinker double-stranded DNA was phosphorylated using T4 polynucleotide kinase. This polylinker was inserted into the cloning sites of pSTA1204 by overnight ligation. DNA clones were first digested with *Bam*HI to confirm the addition of the polylinker. The DNA sequence

of pSTA1210 was then carried out in order to confirm the integrity of these cloning sites. The sequencing primer is homologous to the positive strand of the *amy3* gene located at 50 bp upstream of the stop codon. The results are shown on Figure 3.2.4.

SECTION III:

CONSTRUCTION OF HN SECRETION VECTORS

For several decades viral diseases have been controlled by vaccination programmes using 'killed' or attenuated virus vaccines. The lower immunity obtained by the former and the potential of the latter to become virulent have encouraged the development of an alternative approach. The construction of solid matrix-antibody-antigen (SMAA) complexes may be represent an alternative for effective antiviral vaccines. This strategy consists of solid matrices saturated with specific monoclonal antibodies, which in turn are saturated with their respective antigens. This resulting SMAA complexes are then used as immunogens (Randall, 1989). However, the development of this subunit vaccines requires the production of authentic virus glycoproteins capable of inducing virus neutralizing antibodies at high levels.

The production of authentic viral glycoprotein by *A.oryzae* was investigated. For these studies the

Figure 3.2.4 Sequencing results of pSTA1210 encoding the *A.oryzae amy3* gene. pSTA1210 was incubated in a solution containing 0.2M NaOH, 0.2 mM EDTA, at 37°C for 30 min in order to desnature double strand DNA. To the solution was added 0.1 vol of 3 M NaOAc pH 5.2 and 3 vol 96 % ethanol. Incubation was carried out at -70°C for 15 min. The DNA was pelleted, washed and resuspended in 7 μ l of sterile distilled water. The pSTA1210 was sequenced by the chain-termination sequencing DNA method described in Sanger et al., 1977. The 5'amy primer used is homologous to the amy3 sequences located 50 bp upstream the stop codon. The results confirm the presence and integrity of cloning sites encoding DNA inserted at the natural stop codon of the amy3 gene (see text for details).



Haemagglutinin-neuraminidase (HN) protein from simian virus 5 was used as a model. The advantage of using HN protein is the known aspects of HN protein processing, glycosylation and folding provided by the studies where HN protein was expressed as membrane-bound protein (Paterson *et al.*, 1985; Ng *et al.*, 1990) and secreted protein (Paterson & Lamb, 1990) in cell tissue culture. The strategy used was the fusion of the HN cDNA sequence to a high expressed fungal gene in order to obtain high levels of HN protein secreted in the medium.

3.1 Synthesis of the HN fragment by PCR

In pGEXchNTAG, the HN protein encoding gene is linked at its 3'-terminus to an oligonucleotide encoding the 'tag' antigen (T.Hanke, unpublished). This tag sequence consists of 14 aa of SV5 P&V proteins recognised by the monoclonal antibody SV5-P-k (Southern *et al.*, 1991) which enables the purification of the expressed HN protein. Amplification of the complete HN cDNA and TAG sequences (designated HN-PCR fragment) was performed from pGEXchNTAG by PCR technology. The 5' primer HNback is homologous to the first 22 bp of the positive strand of HN cDNA sequence and at its 5' end has encoded the *EcoRI* and *XbaI* cloning sites. The 3' universal primer GEXFOR is homologous to the negative strand of the cloning vector pGEX sequences downstream

the cloning sites. GEXFOR primer harbours the *HindIII*, *BssHII* and *SalI* sites at its 5' end.

However, as HN is a membrane bound glycoprotein type II, it might not be secreted due to its anchor-domain, thus a deletion of the first 108 bp of its cDNA sequence, corresponding to the hydrophobic N-terminal region, was carried out in a second PCR reaction (ASSHN-PCR fragment). The 5' primer Δ SSHNback used is 44 bp long of which 23 bp is homologous to HN cDNA sequence from position 109. Its 5' end encodes the *EcoRI* and *XbaI* cloning sites (Figure 3.3.1).

Polymerase chain reaction was performed as described in Materials and Methods Section 2.6. Denaturation temperature was 94°C for 1 min and DNA extension temperature was 65°C for 2 min. In the first cycle the annealing temperature was 40°C for 1.5 min, which was changed to 55°C for 1.5 min in the following 30 cycles. The results are shown in Figure 3.3.2.

3.2 Construction of HN expression cassettes

The structure of HN expression cassettes are shown in Figure 3.3.3. Both HN- and ASSHN- PCR fragments were inserted either in pSTA1210 downstream of the *A.oryzae amy3* gene or in pAN56-30 downstream of the *A.niger glaA* coding region.

Figure 3.3.1 Design of primers. GEXFOR primer is homologous to the negative strand of the cloning vector pGEX sequences downstream the cloning sites. Therefore, it can be used, as 3'primer, to amplify any sequence cloned in this plasmid. HNback primer is homologous to the the first 22 bp of the positive strand of HN cDNA sequence. Δ SSHback primer is homologous to the positive strand of HN cDNA sequence between the position 109 and 131 bp. The Δ SSHback was used to carry a deletion in the DNA sequence corresponding to the N-terminal hydrophobic region of HN protein. The red line marks the stop codon. The green lines are shown restriction endonuclease sites. The blue line underlines the KEX2-site.

A - GEXFOR

^{5'} C GCT CTG TCG ACG CGC GCA AGC TTC AGA TCG TGA GTC AGT CAC GAT GAA ^{3'} TT

B - HNback

^{5'} AAC CGA GAA TTC TCT AGA AGG ATG GTT GCA GAA GAT GCC CCT ^{3'} G

C - ΔSSHNback

^{5'} AAC AGA GAA TTC TCT AGA AGG GAG AGT TTA ATA ACC CAA AAG ^{3'} CA

Figure 3.3.2 Polymerase chain reaction (PCR) of the Haemagglutinin-neuraminidase (HN) cDNA sequence.

Panel A - The complete HN cDNA sequence was amplified using the 5'primer HNback and the 3'primer GEXFOR. The conditions were described somewhere in the text.

- Lane 1 - Markers, *Hind*III digested lambda DNA
- Lane 2 - Markers, 100 bp DNA ladder
- Lane 3 - Reaction in absence of GEXFOR primer
- Lane 4 - Reaction in absence of HNback primer
- Lane 5 - Reaction in absence of template DNA
- Lane 6 - Reaction with 1 ng of template DNA
- Lane 7 - Reaction with 10 ng of template DNA
- Lane 8 - Reaction with 100 ng of template DNA
- Lane 9 - pGEXCHNTAG

Panel B - A deletion of the first 108 base pairs which encode the N-terminal hydrophobic region of HN protein was carried out by PCR. HN cDNA sequence was amplified using the 5'primer ASSHNback and the 3'primer GEXFOR.

- Lane 1 - Markers, 100 bp DNA ladder
- Lane 2 - Markers, *Hind*III digested lambda DNA
- Lane 3 - Reaction in absence of primers
- Lane 4 - Reaction in absence of template
- Lane 5 - Reaction with 1 ng of template
- Lane 6 - Reaction with 10 ng of template
- Lane 7 - Reaction with 100 ng of template
- Lane 8 - pGEXCHNTAG

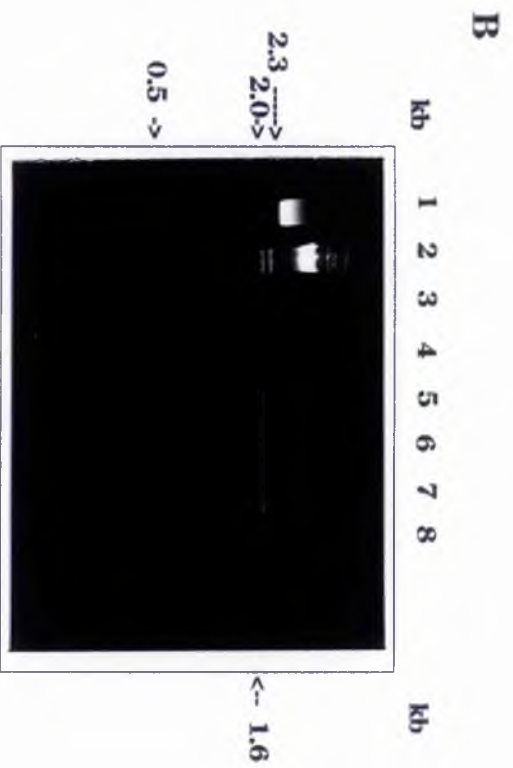
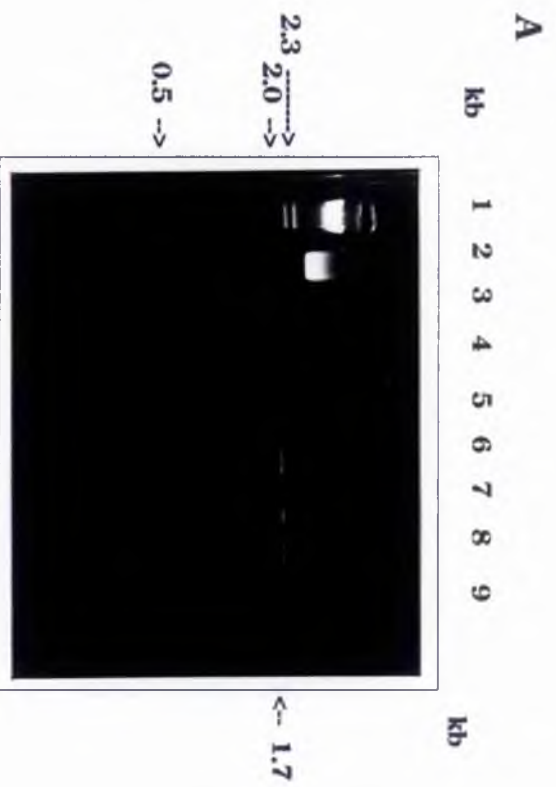
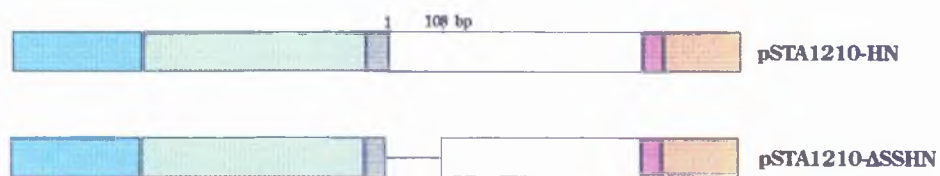
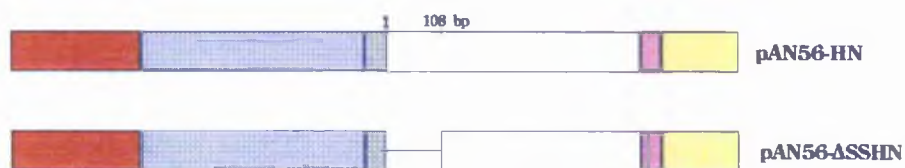











Figure 3.3.3 **Structure of the HN expression cassettes.**
Both HN- and ASSHN-PCR fragments were inserted downstream of the *A.oryzae amy3* gene in pSTA1210 (Panel A), generating pSTA1210-HN and pSTA1210-ASSHN respectively. Both PCR fragments were also inserted downstream of the *A.niger glaA* coding region in pAN56-30 (Panel B), generating pAN56-HN and pAN56-ASSHN respectively.

A) pSTA1210



B) pAN56-30



-  *amy3* coding region
-  HN cDNA sequences
-  *amy3* promoter
-  *amy3* terminator
-  tag sequence
-  Kex2-site
-  *glaA* coding region
-  *gpdA* promoter
-  *trpC* terminator

3.2.1 Inserting HN- and ASSHN-PCR products into pSTA1210

HN- and ASSHN-PCR fragments were digested with an excess of *Xba*I and *Hind*III restriction enzymes (5 U/ μ g DNA), overnight. These fragments were isolated by electrophoresis and purified by glass milk matrix. Ligation between each of these PCR fragments and the dephosphorylated *Xba*I and *Hind*III digested pUC18 vector was carried out at 15°C overnight. The transformed white *E.coli* colonies were selected for plasmid extraction and further digestion with *Xba*I and *Hind*III to recover the inserted fragment (Figure 3.3.4). One clone carrying the entire HN sequence (pUCHN) and another carrying the deleted form of HN (pUCASSHN) were submitted to large scale plasmid extraction and digested with *Xba*I and *Hind*III. A fragment of approximately 1.6 kb from both plasmids (corresponding to the HN viral protein encoding DNA sequence) was purified by electroelution. Ligation reaction between each of these *Xba*I-*Hind*III fragments and the dephosphorylated *Xba*I-*Hind*III digested pSTA1210 vector was performed at 15°C overnight. Plasmid DNA of the clones obtained was extracted and digested with *Xba*I and *Hind*III. The HN DNA insert was recovered from 7 clones out of 24 and ASSHN insert was recovered from 40 clones out of 48 (Figure 3.3.5). pSTA1210-HN and pSTA1210-ASSHN were sequenced using a 19-mer primer homologous to sequences of the positive strand of the *A.oryzae amy3* gene located 50 bp upstream of the stop codon position. The results confirm that the HN- and

Figure 3.3.4 Insertion of HN-PCR fragments into pUC18. Both HN- and ASSHN- PCR fragments were digested with *Xba*I and *Hind*III restriction endonucleases and ligated into pUC18 also digested by the same enzymes. Positive clones were screened by colony coloration among *E.coli* transformed cells.

Panel A - Restriction endonuclease digestion of DNA from white colonies transformed with pUC18-HN ligation reaction. The DNA clones were submitted to DNA mini-preparation followed by double digestion with *Xba*I and *Hind*III restriction endonucleases. The digested DNA was run in a 1 % agarose gel.

- Lane 1 - pUC18 undigestion
- Lane 2 - pUC18 *Xba*I-*Hind*III digestion
- Lane 3 - HN-PCR fragment *Xba*I-*Hind*III digestion
- Lanes 4 to 14 - DNA from transformed *E.coli* colonies
- Lane 15 - Markers, *Hind*III digested lambda DNA

Panel B - Restriction endonuclease digestion of DNA from white colonies transformed with pUC18-ASSHN ligation reaction. The DNA clones were submitted to DNA mini-preparation followed by double digestion with *Xba*I and *Hind*III restriction endonucleases. The digested DNA was run in a 1 % agarose gel.

- Lane 1 - Markers, *Hind*III digested lambda DNA
- Lane 2 - HN-PCR fragment undigestion
- Lane 3 - ASSHN-PCR fragment undigestion
- Lane 4 - pUC18 *Xba*I-*Hind*III digestion
- Lane 5 - pUC18 undigestion
- Lane 6 - HN-PCR fragment *Xba*I-*Hind*III digestion
- Lane 7 - ASSHN-PCR fragment *Xba*I-*Hind*III digestion
- Lanes 8 to 15 - DNA from transformed *E.coli* cells

A



B



Figure 3.3.5 Insertion of HN-PCR fragments into pSTA1210. Both pUC18HN and pUC18ASSHN were digested with *Xba*I and *Hind*III restriction endonucleases. The smallest fragment, of approximately 1.7 kb, was isolated and ligated into pSTA1210 also digested with *Xba*I-*Hind*III restriction endonucleases.

Panel A - Restriction endonuclease digestion of DNA from *E.coli* colonies transformed with pSTA1210-HN ligation reaction. The DNA clones were submitted to DNA mini-preparation followed by double digestion with *Xba*I and *Hind*III restriction endonucleases. The digested DNA was run in a 1 % agarose gel.

Lane 1 - pSTA1210 *Xba*I-*Hind*III digestion
Lanes 2 to 17 - DNA from transformed *E.coli* colonies
Lane 18 - HN-PCR fragment *Xba*I-*Hind*III digestion

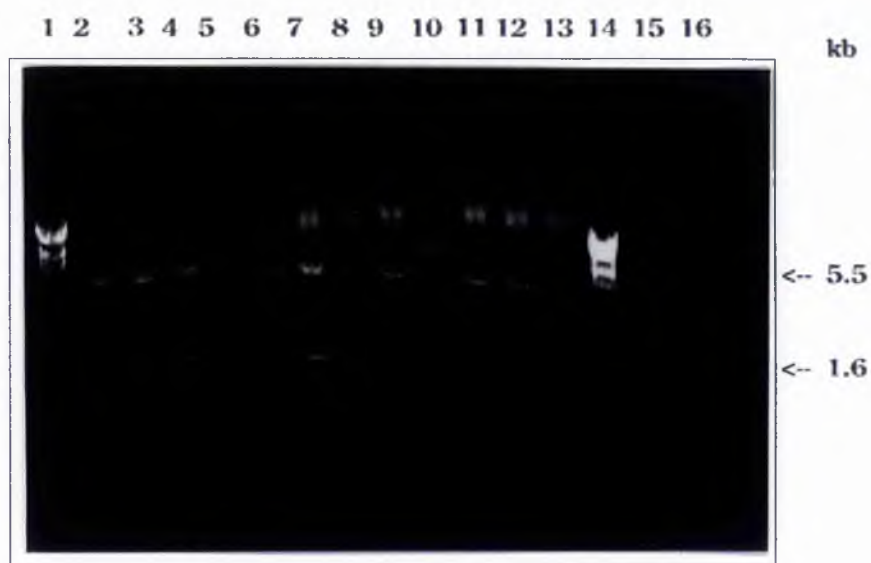
Panel B - Restriction endonuclease digestion of DNA from *E.coli* colonies transformed with pSTA1210-ASSHN ligation reaction. The DNA clones were submitted to DNA mini-preparation followed by double digestion with *Xba*I and *Hind*III restriction endonucleases. The digested DNA was run in a 1 % agarose gel.

Lane 1 - Markers, *Hind*III digested lambda DNA
Lane 2 to 13 - DNA from transformed *E.coli* cells
Lane 14 - Markers, *Hind*III digested lambda DNA
Lane 15 - pSTA1210 *Xba*I-*Hind*III digestion
Lane 16 - ASSHN-PCR fragment *Xba*I-*Hind*III digestion

A



B



ASSHN- fragments were in frame with the *amy3* gene (Figure 3.3.6).

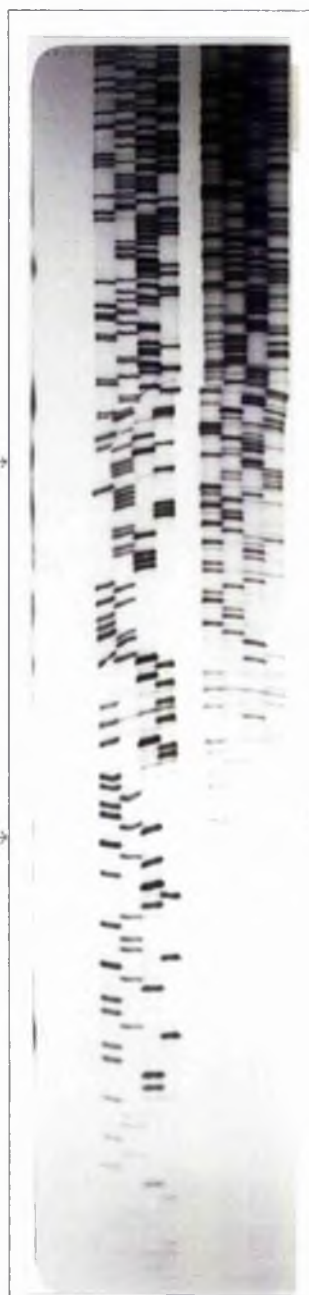
3.2.2 Inserting HN- and ASSHN-PCR products into pAN56-30

pAN56-30 was completely digested with *EcoRI* and dephosphorylated. Additionally, HN- and ASSHN- PCR products were digested with *EcoRI*. Both fragments were purified by electroelution. Ligation between either HN- or ASSHN- fragments and pAN56-30 vector was carried out overnight at 15°C. Positive clones were submitted to plasmid DNA extraction and restriction digestion with *EcoRI* to recover the inserted fragment and with *PstI* to screen for the correct orientation (Figure 3.3.7). Since *EcoRI* digestion of HN- and ASSHN- PCR products excluded the TAG sequence, a further ligation was carried out to replace the 3'end of HN linked to TAG. pAN56-HN3 and pAN56-ASSHN3 clones were digested with *XmaI* and *HindIII*. The largest fragment from each digestion was isolated on 1 % agarose and purified by electroelution. pSTA1210-ASSHN was also digested with *XmaI* and *HindIII* and the smallest fragment of 918 base pairs, which comprises the 3'end of HN cDNA and the TAG sequences was purified by low melting point agarose (Figure 3.3.8) (see Materials and Methods Section 2.5.8.2). Ligation between either the largest *XmaI-HindIII* fragment of pAN56-HN3 or pAN56-ASSH3 and the smallest fragment from pSTA1210-ASSHN restored the carboxyl terminus of HN

Figure 3.3.6 Sequencing gel of pSTA1210HN and pSTA1210ASSHN. Both plasmids were incubated in a solution containing 0.2M NaOH, 0.2 mM EDTA, at 37°C for 30 min in order to desnature double strand DNA. To both solutions were added 0.1 vol of 3 M NaOAc pH 5.2 and 3 vol 96 % ethanol. Incubation was carried out at -70°C for 15 min. Both plasmid DNA were pelleted, washed and resuspended in 7 μ l of sterile distilled water. pSTA1210HN (2) and pSTA1210ASSHN (1) were sequenced by the chain-termination sequencing DNA method described in Sanger et al., 1977. The 5'amy primer was used. This primer is homologous to the amy3 sequences located 50 bp upstream the natural stop codon. The sequence obtained confirmed that in both plasmids, the HN cDNA sequence is in-frame with the amy3 gene.

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Figure 3.3.7 Determination of the right orientation of both HN- and ASSHN- inserts in pAN56-30. HN- and ASSHN- PCR fragments were digested with *EcoRI* restriction endonuclease and ligated into *EcoRI* digested pAN56-30 vector. The plasmid DNA from *E.coli* cells transformed with the ligation mix were digested with *PstI* restriction endonuclease to determine the correct orientation of the insert. The digested DNA was run in a 1 % agarose gel.

Panel A - Analysis of pAN56-HN clones. The *PstI* restriction digestion produces fragments of size marked on the left side, if the fragment was inserted in correct orientation, and on the right side, if the orientation was incorrect.

Lane 1 - Markers, *HindIII* digested lambda DNA
Lanes 2 to 9 - DNA from pAN56-HN clones

Panel B - Analysis of pAN56-ASSHN clones. The *PstI* restriction digestion produces fragments of size marked on the left side, if the fragment was inserted in correct orientation, and on the right side, if the orientation was incorrect.

Lane 1 - Markers, *HindIII* digested lambda DNA
Lanes 2 to 9 - DNA from pAN56-ASSHN clones

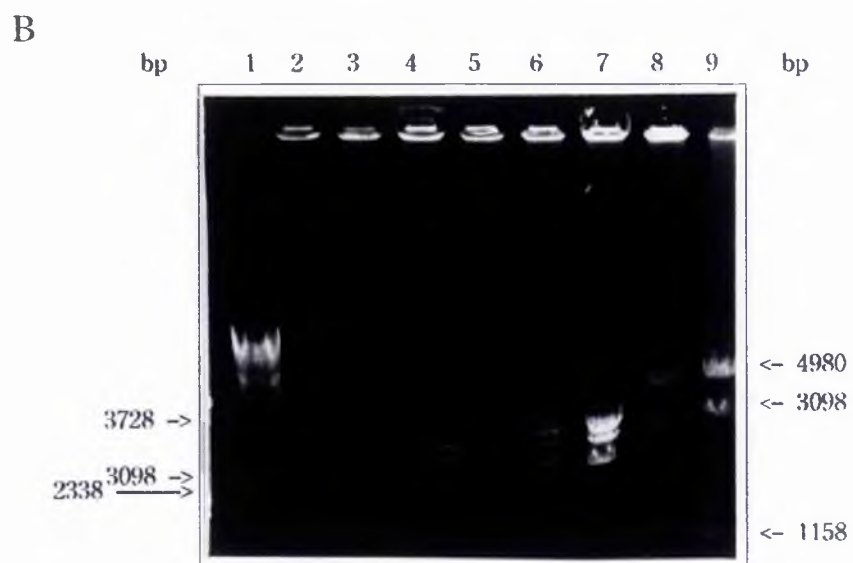
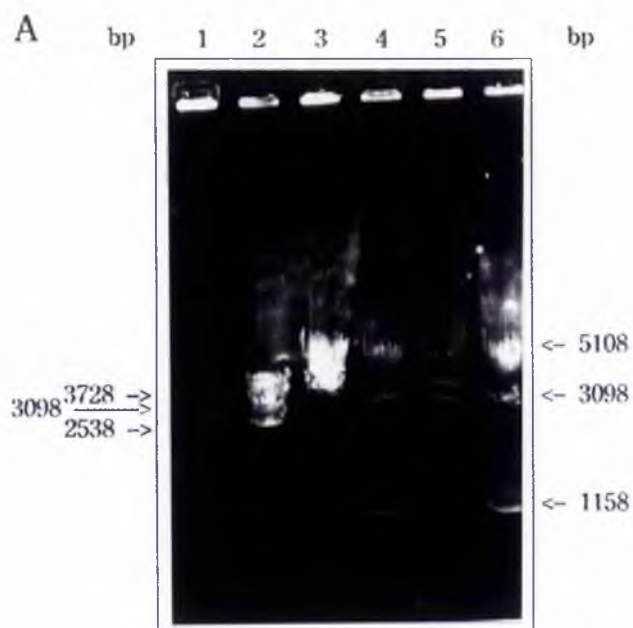


Figure 3.3.8 Restriction endonuclease analysis of both pAN56-HN and pAN56-ASSHN vectors for replacement of the 3'end of HN cDNA and TAG sequences.

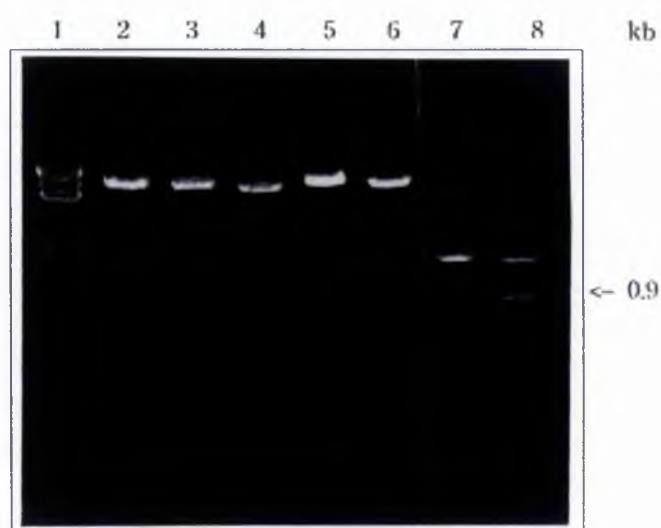
Panel A - Restriction endonuclease digestion of both pAN56-HN and pAN56-ASSHN.

- Lane 1 - Markers, *Hind*III digested lambda DNA
- Lane 2 - pAN56-HN *Hind*III digestion
- Lane 3 - pAN56-HN *Xma*I digestion
- Lane 4 - pAN56-HN *Hind*III-*Xma*I digestion
- Lane 5 - pAN56-ASSHN *Xma*I digestion
- Lane 6 - pAN56-ASSHN *Hind*III- *Xma*I digestion
- Lane 7 - pSTA1210 *Xma*I digestion
- Lane 8 - pSTA1210 *Hind*III-*Xma*I digestion

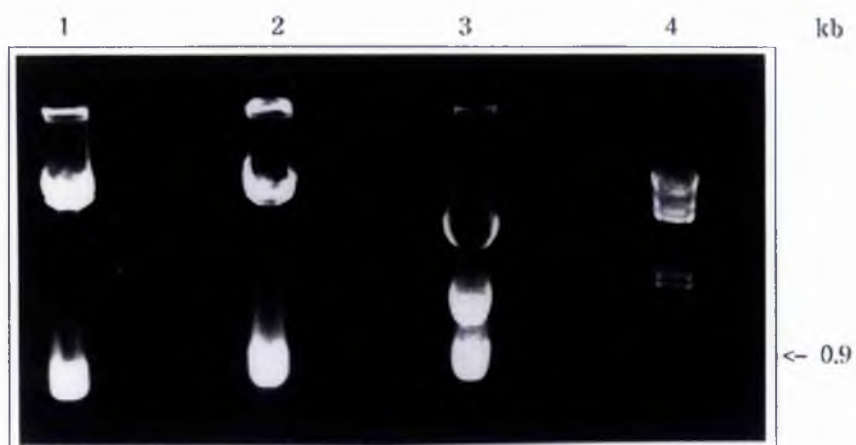
Panel B - A further restriction endonuclease digestion of both pAN56-HN and pAN56-ASSH and pSTA1210. The smallest fragment of either pAN56-HN or pAN56-ASSHN were replaced by the smallest fragment of pSTA1210, which contains the 3'end HN cDNA sequence linked to TAG.

- Lane 1 - pAN56-HN *Hind*III-*Xma*I digestion
- Lane 2 - pAN56-ASSHN *Hind*III-*Xma*I digestion
- Lane 3 - pSTA1210 *Hind*III-*Xma*I digestion
- Lane 4 - Markers, *Hind*III digested lambda DNA

A



B



protein linked to TAG. pAN56-HN1 and pAN56-ASSHN5 were used in later experiments.

Finally, pSTA1210-HN, pSTA1210-ASSHN, pAN56-HN1 and pAN56-ASSHN5 were submitted to PCR using the same primer used to amplify both the HN- and ASSHN- fragments in order to examine their integrity. All the fragments generated were similar in size (Figure 3.3.9).

SECTION IV:

EXPRESSION OF HN VIRAL PROTEIN IN *A.oryzae*

4.1 Genetic Transformation

HN-containing expression vectors were introduced into *Aspergillus oryzae* recipient strains by means of cotransformation experiments. Two selection system were used: arginine prototrophy and acetamide utilisation.

4.1.1 The *argB* selection system

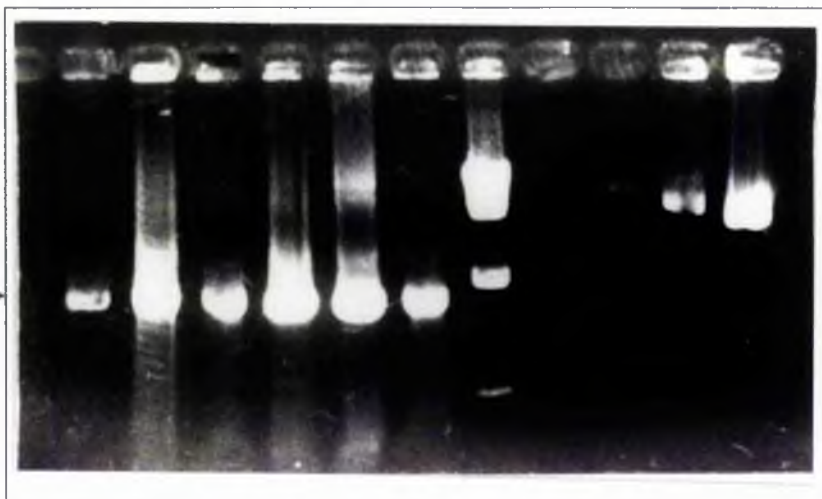
A.oryzae recipient strain 1560-6 was grown in liquid MM supplemented with 1 M sucrose (market sugar) at 30°C for 14 h. Protoplasts were obtained as described in Materials and Methods Section 2.7.1.2. Co-transformation was carried out using pSTA4, which carries the *A.nidulans argB* gene, and one of the HN cDNA- encoding plasmids as shown in Table 3.4.1. 1 µg of

Figure 3.3.9 Polymerase chain reaction (PCR) analysis of HN cDNA containing-expression vectors. HN- and ASSHN-fragments were amplified using 5'HNback and 5'ASSHNback primers respectively. The 3'GEXFOR primer was used in all reactions. The reaction conditions are described in the text. 5 % of the each reaction volume was run in a 1 % of agarose gel.

- Lane 1 - HN fragment amplified from pAN56-HN
- Lane 2 - HN fragment amplified from pSTA1210-HN
- Lane 3 - HN fragment amplified from pGEXCHNTAG
- Lane 4 - ASSHN fragment amplified from pAN56-ASSHN
- Lane 5 - ASSHN fragment amplified from pSTA1210-ASSHN
- Lane 6 - ASSHN fragment amplified from pGEXCHNTAG
- Lane 7 - Markers, *Hind*III digested lambda DNA
- Lane 8 - pAN56-HN DNA
- Lane 9 - pAN56-ASSHN DNA
- Lane 10 - pSTA1210-HN DNA
- Lane 11 - pSTA1210-ASSHN DNA

kb 1 2 3 4 5 6 7 8 9 10 11

1.7 -->



selective plasmid was used in each experiment. The molar concentration of pSTA1210-HN and -ASSHN non-selective plasmids were both seven-fold higher and the molar concentration of pAN56-HN and -ASSHN non-selective plasmids used were both eleven-fold higher. The arginine prototrophic transformants harbouring the HN expression cassettes were screened by genomic DNA dot blot hybridization using the *Bam*HI fragment within the HN DNA sequence as a probe (Figure 3.4.1). Results are shown in Table 3.4.1.

Table 3.4.1 Transformation frequency of *A.oryzae* strain 1560-6 using the *argB* selection system.

Plasmid	Number ^a	Frequency ^b	Cotransformation ^c
pSTA4	18	18	NA
pSTA4 pSTA1210HN	21	21	33 (7)
pSTA4 pSTA1210ASSHN	34	34	38 (13)
pSTA4	17	17	NA
pSTA4 pAN56-HN	22	22	59 (13)
pSTA4 pAN56-ASSHN	12	12	58 (7)

NA, denotes not applicable

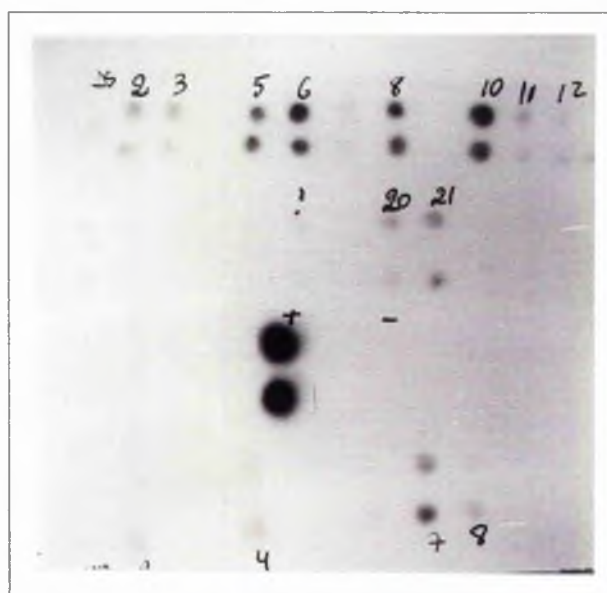
^a Number of transformants obtained

^b Frequency is the number of transformants obtained per 1 μ g of selective plasmid used

^c Co-transformation frequency with non-selective plasmid is expressed as a percentage

The number of HN- transformants is shown in brackets

Figure 3.4.1 Determination of HN cDNA sequence containing-transformants. Approximately 1 μ g of genomic DNA from the arginine prototrophic transformants was dot blotted onto nylon membrane. DNA hybridization was carried out using a *Bam*HI fragment of HN cDNA sequence as a probe, under stringent condition. Genomic DNA from *A.oryzae* strain 1560-6 recipient strain was used as the negative control (-). pGEXCHNTAG was used as the positive control (+).



An increase of six-fold in transformation frequency was observed when comparing the results shown in Table 3.4.1 with those values obtained from previous transformations using pSTA4 as the selective plasmid for arginine selection (see Results Section 1.1.2), which might be due improvements in the technique. It was noticed that mycelia grown in the presence of market sugar yield more transformants / μg of selective DNA used. The co-transformation frequency, which represents the percentage of the arginine selected transformants that contain also the HN expression cassette, was found to be approximately 36 % for both pSTA1210-HN expression vectors and 59 % for both pAN56-HN expression vectors. This difference in co-transformation percentage might be related to the higher molar concentration of the pAN56-HN non-selective plasmids used in transformation.

Transformants containing HN and ASSHN cDNA sequences under the control of the *A.oryzae* amy3 promoter were designated T1560amyHN and T1560amyASSHN respectively and those under the control of the *A.nidulans* gpdA promoter were designated T1560gpdglaHN and T1560gpdglaASSHN respectively.

4.1.2 The *amdS* selection system

An *A.oryzae* *amdS*⁻ mutant, given to Dr Kinghorn by Dr Gomi (Tokyo, Japan) was used as recipient strain. The *amdS*⁻ mutant was grown in liquid MM supplemented with

1 M sucrose (market sugar) at 30°C for 14 h. Protoplasts were obtained as described in Materials and Methods Section 2.7.1.2. Co-transformation was carried out using p3SR2 as the selective plasmid, which contains the *A.nidulans amdS* gene and either pSTA1210-HN or pSTA1210-ASSHN (Table 3.4.2). 2 µg of the selective plasmid was used in each experiment and the molar concentration of pSTA1210-HN and -ASSH nonselective plasmids used were five-fold higher. Transformants were selected for growth on MM containing 2 mM acetamide as the sole nitrogen source, augmented with 15 mM caesium chloride to reduced the background. Transformants with large colony formation and showing signs of sporulation were selected for further studies. The acetamide prototrophic transformants which contain HN expression cassettes were screened by genomic DNA dot blot hybridization as shown in Figure 3.4.2. Results are shown in Table 3.4.2.

Table 3.4.2 Transformation frequency of *A.oryzae* strain Δ 537 using the *amdS* selection system.

Plasmid	Number ^a	Frequency ^b	Cotransformation ^c
p3SR2	34	17	NA
p3SR2 pSTA1210HN	46	23	48 (22)
p3SR2 pSTA1210ASSHN	71	35.5	61 (43)

NA Not applicable

^a Number of transformants obtained

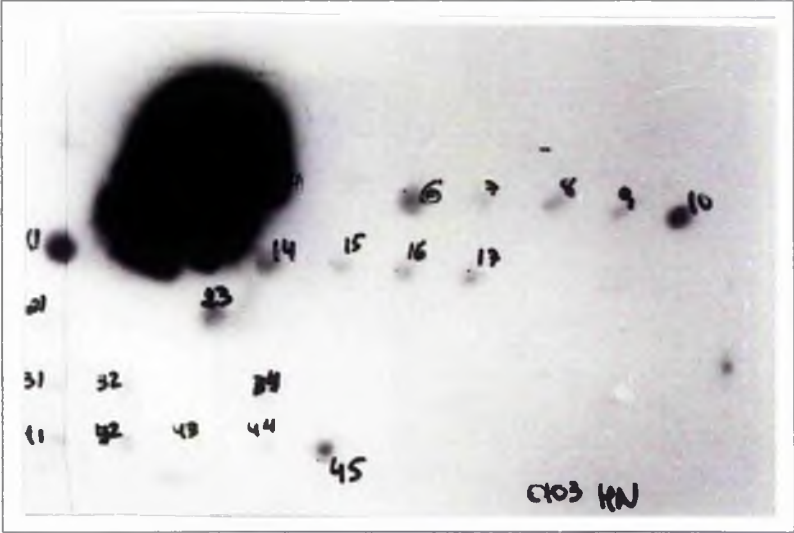
^b Frequency is the total number of transformants obtained per 1 µg of selective plasmid used

^c Co-transformation with non-selective plasmid is expressed as a percentage.

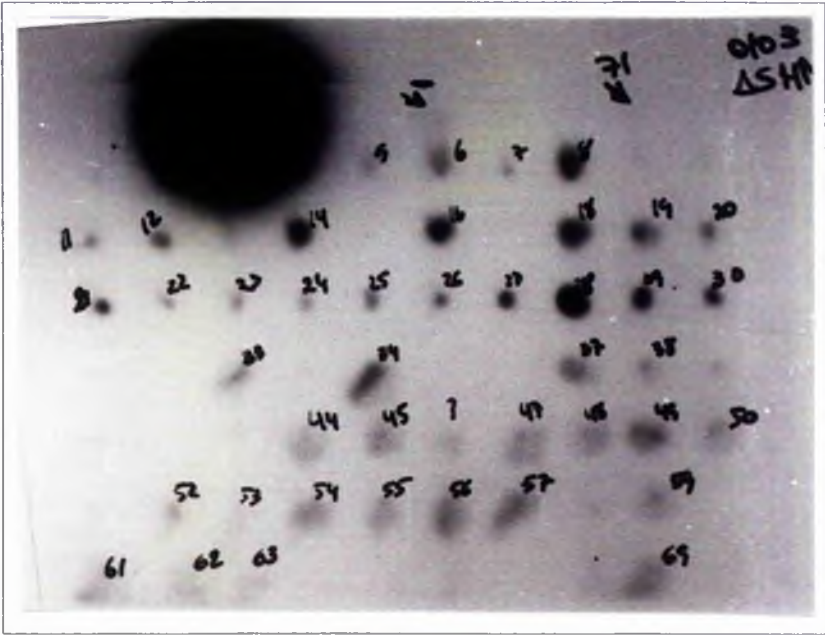
The number of HN-transformants is shown in brackets

Figure 3.4.2 Determination of HN cDNA sequence containing-transformants. Approximately 1 μ g of genomic DNA from the acetamide prototrophic transformants was dot blotted onto nylon membrane. DNA hybridization was carried out using a *Bam*HI fragment of HN cDNA sequence as a probe, under stringent condition. Genomic DNA from *A.oryzae* Δ 537 recipient strain was used as the negative control (-). pGEXCHNTAG was used as the positive control (strong signal). The transformants T537amyHN10 (top picture) and T537amyASSHN18 (bottom picture) were selected and used in later experiments.

A



B



It was found that by using a *amdS*⁻ mutant as the host, background growth discussed in Results Section 1.1.3 was reduced and in fact, insignificant. Furthermore, transformants were larger and showed signs of sporulation within 5 days of incubation. The transformation frequency was similar to that obtained using the arginine system (pSTA4 was used as the selective plasmid). The percentage of co-transformation was found to be about 55 %. HN- and ASSHN-transformants, selected by acetamide utilization, were designated T537amyHN and T537amyASSHN respectively. The prototrophic transformants used in this Section and their features are listed in Table 3.4.3.

4.2 HN detection

Spores from five day-old cultures of HN- and ASSHN-transformants were inoculated in 50 ml MM containing 1 % maltose as the sole carbon source. Maltose was used to induce the HN synthesis by inducing the synthesis of either glucoamylase or amylase (Shibuya et al., 1990; Tomomura et al., 1961). Such cultures were incubated at 34°C for 48 h, with agitation. 1 ml aliquots of culture filtrates were placed into Eppendorf tubes and lyophilized overnight. The resulting powder obtained was used in SDS-PAGE and western blot analysis.

The result of the SDS-PAGE analysis of arginine prototrophic transformants containing HN- and ASSHN-

Table 3.4.3 Criteria for designation of *Aspergillus oryzae* transformants harbouring the HN DNA sequence. HN-transformant were named based on the recipient strain and the structure of the expression cassette used, i.e. the encoded fungal gene and HN DNA sequence .

Strains (a)	Selection (b) systems	Vectors (c)		Expression (d) cassettes
		selective	non-selective	
T537amyHN	acetamide	p3RS2	pSTA1210HN	amylase - HN
T537amyASSHN	acetamide	p3RS2	pSTA1210ASSHN	amylase - ASSHN
T1560amyHN	arginine	pSTA4	pSTA1210HN	amylase - HN
T1560amyASSHN	arginine	pSTA4	pSTA1210ASSHN	amylase - ASSHN
T1560gpdglaHN	arginine	pSTA4	pAN56HN	glucoamylase - HN
T1560gpdglaSSHN	arginine	pSTA4	pAN56ASSHN	glucoamylase - ASSHN

- (a) (b) Transformant number follows this general denomination. Transformants of *A.oryzae* strain A537, an *amdS*⁻ mutant strain, were selected by acetamide utilisation. Transformants of *A.oryzae* strain 1560-6, an *argB*⁻ mutant strain, were selected by arginine utilisation.
- (c) Plasmid p3RS2 encodes the *A.nidulans amdS* gene. Plasmid pSTA4 encodes the *A.nidulans argB* gene. For details of the non-selective plasmid see Results, Section 3.
- (d) Transformant designation included amy or gpdgla for integrated expression cassettes encoding the *A.oryzae amy3* gene or *A.niger glaA* gene respectively. Finally, HN or ASSHN indicates that the integrated expression cassettes encode or the entire HN DNA sequence or a 108 bp deleted HN DNA sequence comprising its hydrophobic N-terminus respectively (see text for details).

sequences is shown in Figures 3.4.3 and 3.4.4 respectively. The estimated molecular weights of the unglycosylated form of ASSHN and HN proteins are approximately 59 kDa and 63 kDa respectively, which might be difficult to identify on SDS-PAGE gel, as the glycosylated forms of *A.oryzae* amylase and glucoamylase are 55 kDa and 66 kDa in size, respectively. Nevertheless, transformed strains with the glucoamylase-HN construct showed a wide band of relative mobility of approximately 100 kDa, which corresponds to the *A.niger* glucoamylase. This finding indicated that the expression cassettes had been transcribed and translated. However, only T1560amyASSHN-18, -21, and T1560gpdglaASSHN-3 showed a weak signal on a western blot which suggested degradation of HN protein (Lucena, unpublished).

Figures 3.4.5 and 3.4.6 show SDS-PAGE analysis of acetamide prototrophic T537amyHN and T537amyASSHN transformants. Unfortunately, the western blots of these gels probed with anti-TAG MAb were negative (Lucena, unpublished). Nevertheless, transformants T537amyHN-10 and T537amyASSHN-18 were selected for further studies on basis of the positive hybridization (Figure 3.4.2).

A different mycelial morphology was also observed in liquid culture. There were transformants growing in small pellets, while others grew as a block of an amorphous mass. Moreover, T1560amyASSHN-2 and T1560amyHN-12 showed very poor growth in liquid culture, but satisfactory growth on agar media. We speculated

Figure 3.4.3 SDS-polyacrylamide gel analysis of arginine prototrophic transformants which contain the HN fragment incorporated into their genomic DNA. Transformants were grown in MM containing 1 % maltose as the sole carbon source and 10 mM ammonium as the sole nitrogen source, at 34°C, for 48 h. Proteins contained in 1 ml of culture filtrates were separated in a 12.5 % SDS-polyacrylamide gel. The gel was stained with Coomassie blue and destained with methanol-acetic acid solution. Proteins from *A.oryzae* 1560-6 recipient strain filtrate were loaded on gel. Results are shown in Figure 5.4. The arrow on the right shows the *A.niger* glucoamylase band position.

Lane M - Molecular weight markers, sizes are in kDa
Lane 1 - Transformant T1560amyHN-2
Lane 2 - Transformant T1560amyHN-4
Lane 3 - Transformant T1560amyHN-7
Lane 4 - Transformant T1560amyHN-8
Lane 5 - Transformant T1560amyHN-12
Lane 6 - Transformant T1560amyHN-16
Lane 7 - Transformant T1560amyHN-17
Lane 8 - Transformant T1560gpdglaHN-15
Lane 9 - Transformant T1560gpdglaHN-12
Lane 10 - Transformant T1560gpdglaHN-11
Lane 11 - Transformant T1560gpdglaHN-8
Lane 12 - Transformant T1560gpdglaHN-7
Lane 13 - Transformant T1560gpdglaHN-6
Lane 14 - Transformant T1560gpdglaHN-2

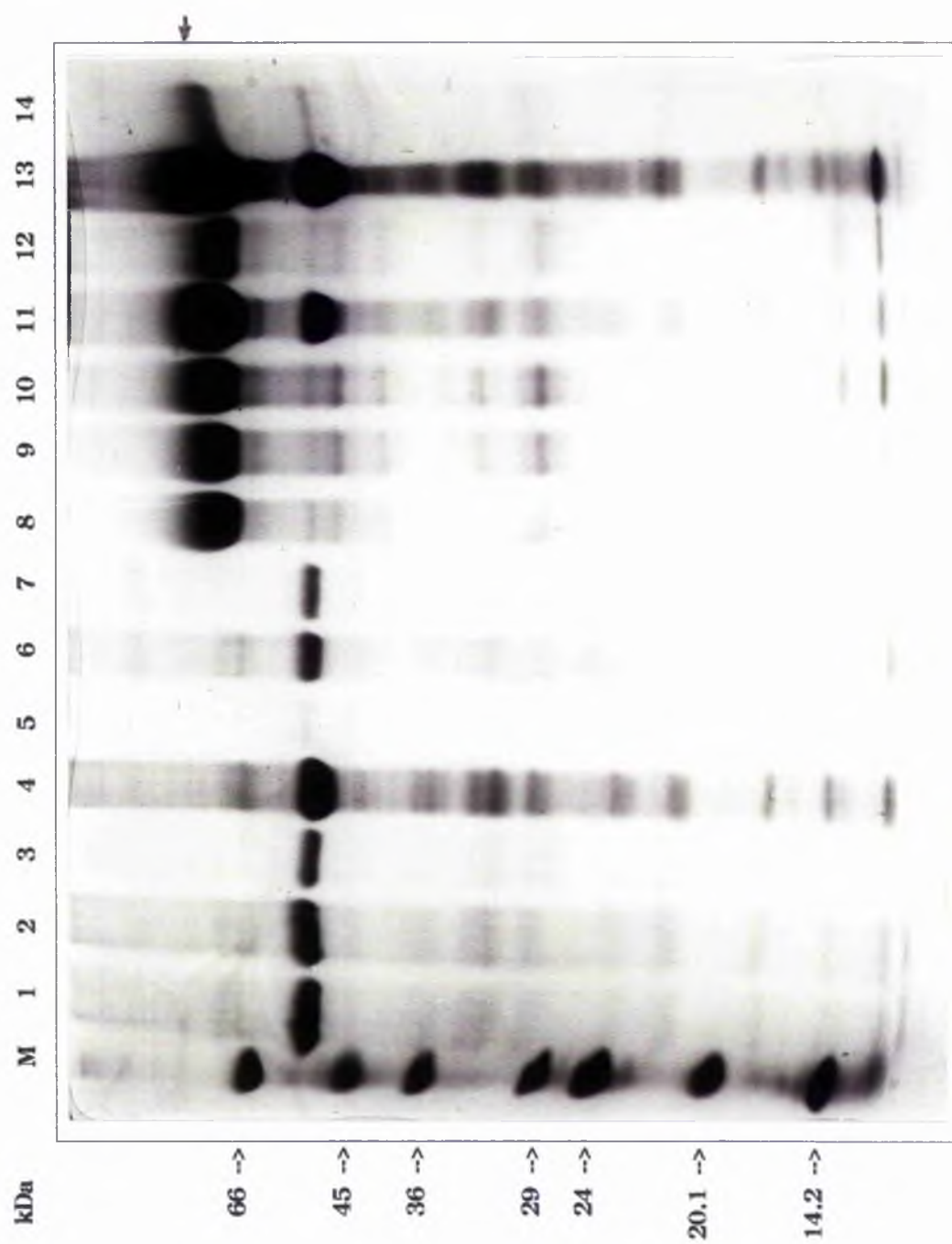


Figure 3.4.4 SDS-polyacrylamide gel analysis of arginine prototrophic transformants which contain the ASSHN fragment incorporated into the genomic DNA. Transformants were grown in MM containing 1 % maltose as the sole carbon source and 10 mM ammonium as the sole nitrogen source, at 34°C, for 48 h. Proteins contained in 1 ml of culture filtrates were separated in a 12.5 % SDS-polyacrylamide gel. The gel was stained with Coomassie blue and destained with methanol-acetic acid solution. The arrow on the right shows the *A.niger* glucoamylase band position.

Lane M - Molecular weight markers, sizes are in kDa
Lane C - Desnaturated HN immunocomplex
Lane 1 - *A.oryzae* strain 1560-6, recipient strain
Lane 2 - Transformant T1560amyASSHN-2
Lane 3 - Transformant T1560amyASSHN-3
Lane 4 - Transformant T1560amyASSHN-5
Lane 5 - Transformant T1560amyASSHN-6
Lane 6 - Transformant T1560amyASSHN-8
Lane 7 - Transformant T1560amyASSHN-10
Lane 8 - Transformant T1560amyASSHN-11
Lane 9 - Transformant T1560amyASSHN-12
Lane 10 - Transformant T1560amyASSHN-18
Lane 11 - Transformant T1560amyASSHN-20
Lane 12 - Transformant T1560amyASSHN-21
Lane 13 - Transformant T1560amyASSHN-34
Lane 14 - Transformant T1560gpdglaASSHN-3

kDa

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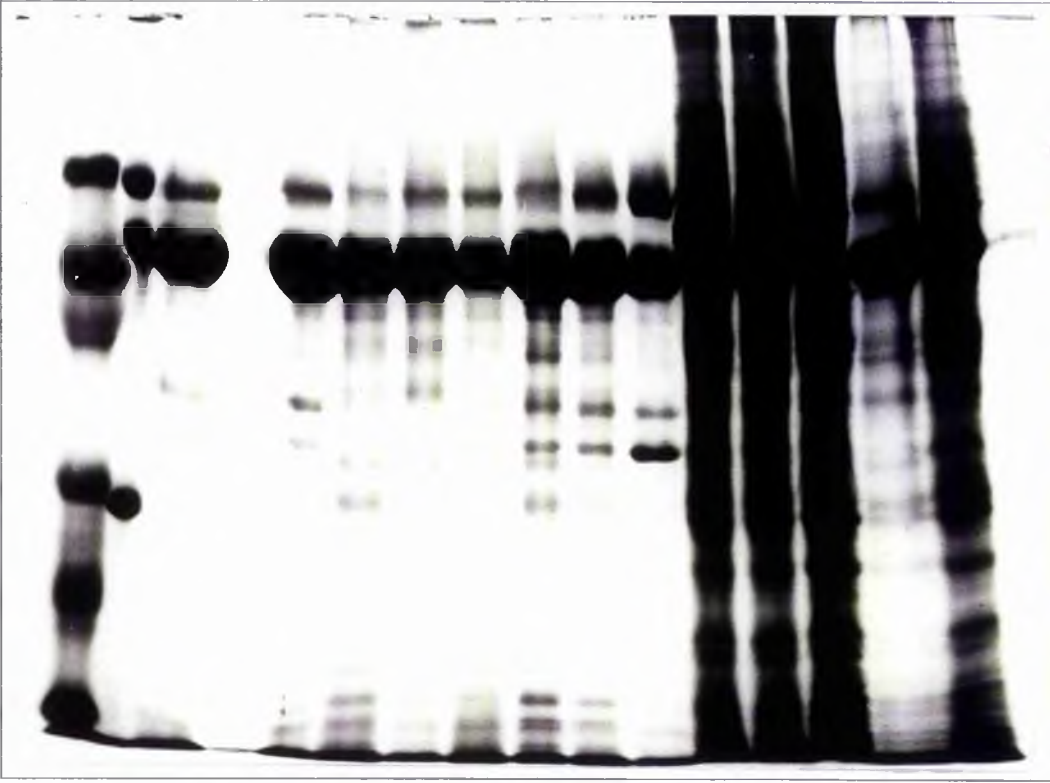


Figure 3.4.5 SDS-polyacrylamide gel analysis of acetamide prototrophic transformants which contain the HN fragment incorporated into the genomic DNA. Transformants were grown in MM containing 1 % maltose as the sole carbon source and 10 mM ammonium as the sole nitrogen source, at 34°C, for 48 h. Proteins contained in 1 ml of culture filtrates were separated in a 12.5 % SDS-polyacrylamide gel. The gel was stained with Coomassie blue and destained with methanol-acetic acid solution.

Lane M - Molecular weight markers, sizes are in kDa
Lane C - Desaturated HN immunocomplex
Lane 1 - *A.oryzae* strain Δ 537, recipient strain
Lane 2 - Transformant T537amyHN-4
Lane 3 - Transformant T537amyHN-6
Lane 4 - Transformant T537amyHN-8
Lane 5 - Transformant T537amyHN-10
Lane 6 - Transformant T537amyHN-11
Lane 7 - Transformant T537amyHN-13
Lane 8 - Transformant T537amyHN-14
Lane 9 - Transformant T537amyHN-16
Lane 10 - Transformant T537amyHN-17
Lane 11 - Transformant T537amyHN-23
Lane 12 - Transformant T537amyHN-45
Lane 13 - Transformant T537amyASSHN-57
Lane M - Molecular weight markers, sizes are in kDa

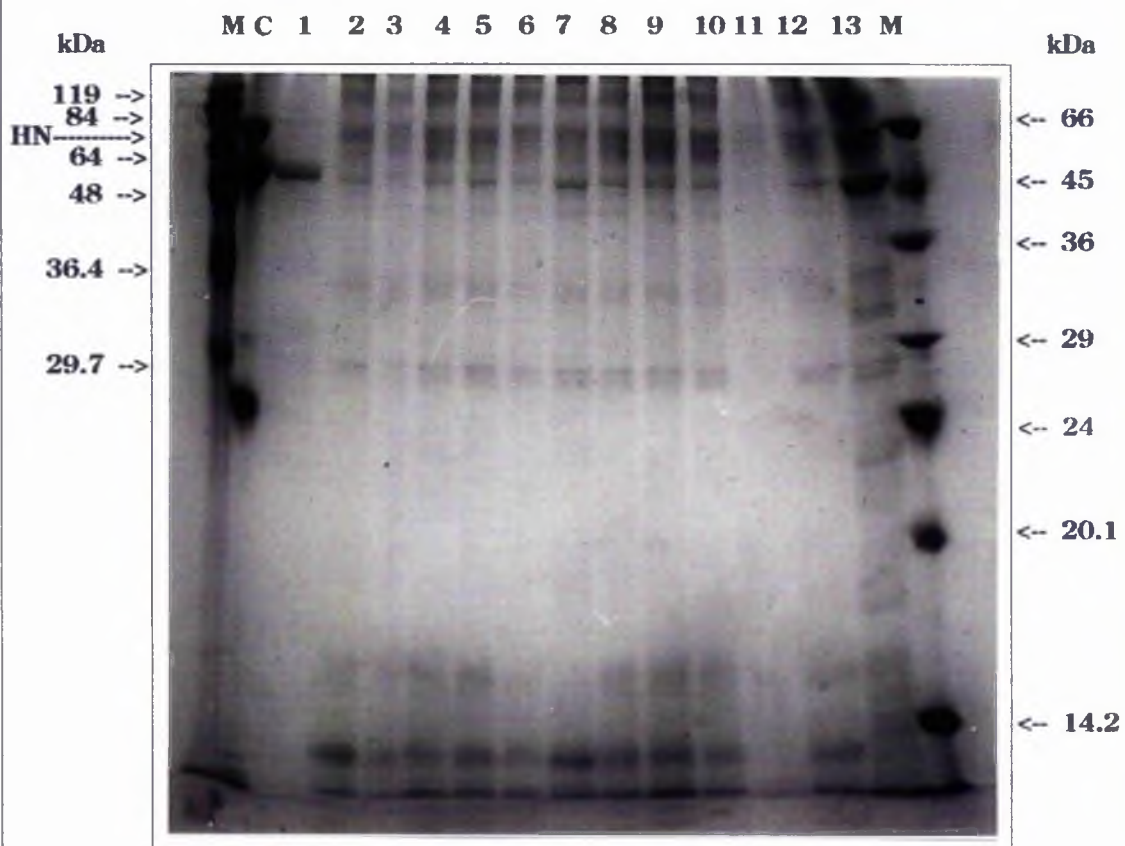
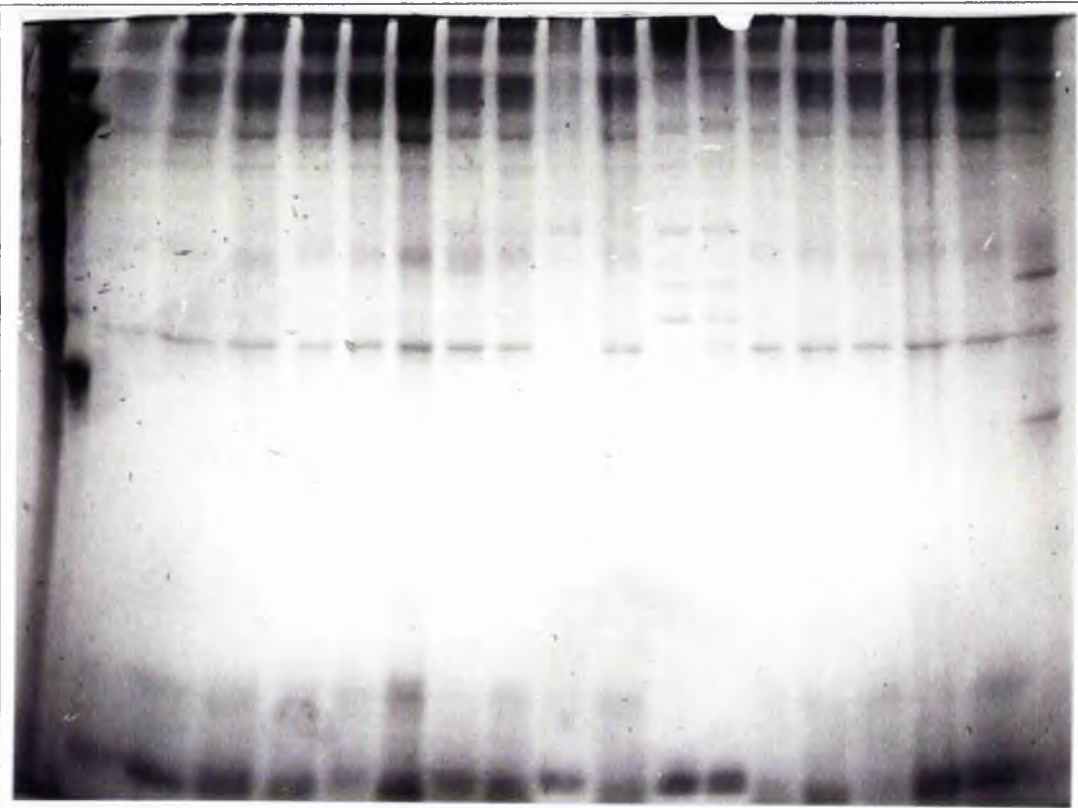


Figure 3.4.6 SDS-polyacrylamide gel analysis of acetamide prototrophic transformants which contain the ASSHN fragment incorporated into the genomic DNA. Transformants were grown in MM containing 1 % maltose as the sole carbon source and 10 mM ammonium as the sole nitrogen source, at 34°C for 48 h. Proteins contained in 1 ml of culture filtrates were separated in a 12.5 % SDS-polyacrylamide gel. The gel was stained with Coomassie blue and destained with methanol-acetic acid solution. Proteins from the *A.oryzae* Δ 537 recipient strain filtrate were loaded on the gel. The results are shown in Figure 4.5.

Lane M - Molecular weight markers, sizes are in kDa
Lane C - Denatured HN immunocomplex
Lane 1 - Transformant T537amyASSHN-6
Lane 2 - Transformant T537amyASSHN-8
Lane 3 - Transformant T537amyASSHN-12
Lane 4 - Transformant T537amyASSHN-14
Lane 5 - Transformant T537amyASSHN-16
Lane 6 - Transformant T537amyASSHN-18
Lane 7 - Transformant T537amyASSHN-19
Lane 8 - Transformant T537amyASSHN-20
Lane 9 - Transformant T537amyASSHN-21
Lane 10 - Transformant T537amyASSHN-26
Lane 11 - Transformant T537amyASSHN-27
Lane 12 - Transformant T537amyASSHN-28
Lane 13 - Transformant T537amyASSHN-29
Lane 14 - Transformant T537amyASSHN-30
Lane 15 - Transformant T537amyASSHN-33
Lane 16 - Transformant T537amyASSHN-35
Lane 17 - Transformant T537amyASSHN-49
Lane 18 - Transformant T537amyASSHN-56

kDa	M	C	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
119 -->																				
HN ----->																				
64 -->																				
48 -->																				
36.4 -->																				
29.7 -->																				



that this altered growth behaviour might be related to the HN expression, HN being toxic to *A.oryzae* cells. Therefore, in order to minimize the degradation of HN protein in the liquid culture by *A.oryzae* extra-cellular proteases thereby allowing detection of mature HN protein, and also, to study the toxicity of HN protein on *A.oryzae* growth, a time course of HN production was carried out. For this study we selected one *glaA*-HN DNA containing transformant (T1560gpdglaASSHN-3) and one *amy3*-HN DNA containing transformant (T1560amyASSHN-18).

4.3 Time course of HN production by transformant T1560amyASSHN-18

10^8 spores of transformant T1560amyASSHN-18 were inoculated into 10 vials of 50 ml MM containing 1 % glucose as the sole carbon source. These cultures were incubated at 34°C, with agitation, for 12-14 h. Mycelia from 5 vials were washed with 1 % maltose MM and reinoculated into fresh MM containing 1 % maltose as the sole carbon source (inducing condition for HN synthesis), while the five other vials were washed with 1 % glucose MM and reinoculated into fresh MM containing 1 % glucose as the sole carbon source (non-inducing condition, control). All cultures were reincubated as before. One culture from each condition were harvested after 1.5, 3, 6, 9 and 24 h of incubation. Two 1 ml aliquots of these culture filtrates were lyophilized.

The resulting powder obtained was used in SDS-PAGE analysis and western blotting. Protease inhibitors (PMSF and EDTA to 1 mM final concentration) were added to the remainder of each culture filtrate (about 48 ml). Immunoprecipitation was carried out using anti-TAG MAb at 4°C for 4 h (See Materials and Methods Section 2.8.4). Immunocomplexes were loaded onto a SDS-PAGE gel and subsequently transferred onto nitrocellulose membrane. The filter was probed with anti-TAG MAb and protein A conjugated to peroxidase.

SDS-PAGE results from the T1560amyASSHN-18 culture grown in non-induced conditions suggested that amylase was produced constitutively by *A.oryzae*, reaching a plateau within 6 h of growth. Maltose induced amylase synthesis which seemed to reach the maximum of secretion after 9 h of growth in these inducing conditions. However, no bands were visualized on SDS-PAGE gel which may be the HN protein (Figure 3.4.7, Panel A). Western blot of proteins from 1 ml culture filtrate probed with anti-TAG MAb failed to detect HN protein, though the TAG-labelled protein marker was visualized (Lucena, unpublished). However, HN was detected on the immunoblot from the immunoprecipitation samples (Figure 3.4.7, Panel B). Under non-induced conditions, HN protein was detected within 3 h of growth, reached the maximum in 6 h, and after 24 h of growth, no HN protein was detected. Under induced conditions, the HN protein was detected earlier and in higher levels than in non-induced

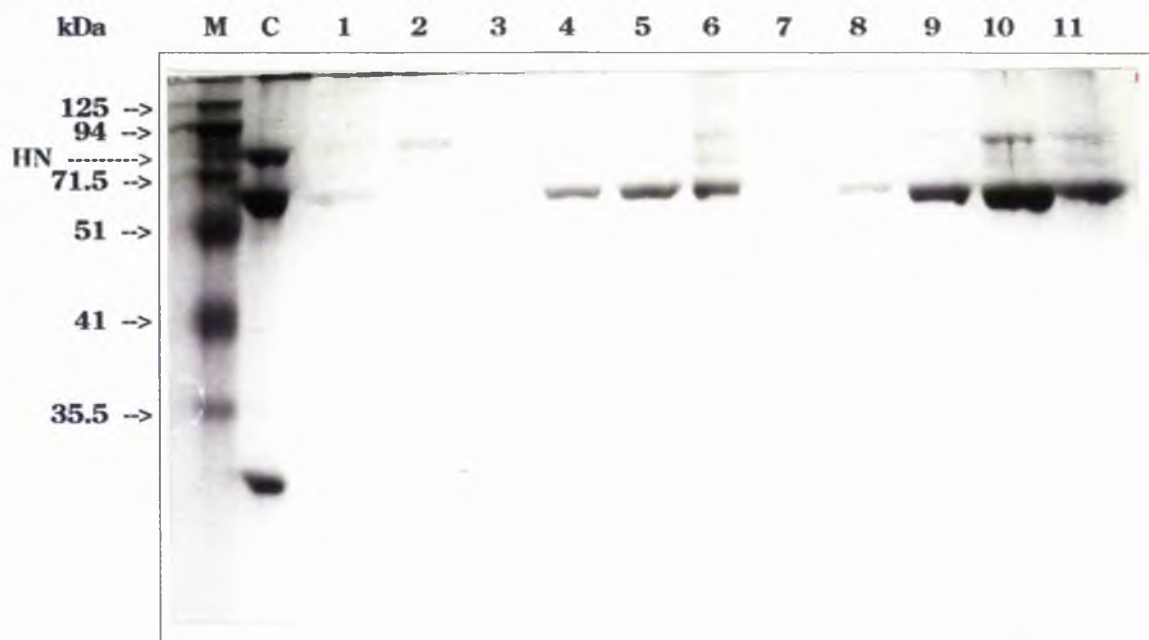
Figure 3.4.7 Time course of HN production by T1560amyASSHN-18 transformant. T1560amyASSHN-18 strain were grown in MM containing glucose 1 % as the sole carbon source and ammonium as the sole nitrogen source, at 34°C, overnight. Mycelia were harvested and reinoculated into fresh MM containing either glucose 1 % or maltose 1 % as the sole carbon source. Cultures were harvested after 1.5, 3, 6, 9, and 24 h of incubation at 34°C, with agitation.

Panel A - SDS-polyacrylamide gel analysis. Proteins from 1 ml culture filtrates were separated in a 12.5 % SDS-PAGE, using descontinuos buffer. The gel was stained with Coomassie blue and destained with methanol-acetic acid solution.

Panel B - Proteins immunoprecipitated with anti-TAG MAb were western blotted and probed with anti-TAG MAb and protein A peroxidase conjugated. HN protein corresponding band was detected by ECL reagents.

- Lane M - Molecular weight markers, sizes are in kDa
- Lane C - Denatured HN immunocomplex (SDS-PAGE) or tag-labelled protein marker (western blot).
- Lane 1 - T1560amyASSHN-18 grown in glucose, overnight
- Lane 2 - T1560amyASSHN-18 grown in glucose, for 1.5 h
- Lane 3 - T1560amyASSHN-18 grown in glucose, for 3 h
- Lane 4 - T1560amyASSHN-18 grown in glucose, for 6 h
- Lane 5 - T1560amyASSHN-18 grown in glucose, for 9 h
- Lane 6 - T1560amyASSHN-18 grown in glucose, for 24 h
- Lane 7 - T1560amyASSHN-18 grown in maltose, for 1.5 h
- Lane 8 - T1560amyASSHN-18 grown in maltose, for 3 h
- Lane 9 - T1560amyASSHN-18 grown in maltose, for 6 h
- Lane 10 - T1560amyASSHN-18 grown in maltose, for 9 h
- Lane 11 - T1560amyASSHN-18 grown in maltose, for 24 h

A



B



conditions. HN was detected within 1.5 h after induction, reaching a plateau in 6 h and, after 24 h of induction the level of HN protein was still significant. This finding suggested that if HN has a degree of toxicity towards the *A. oryzae* cell, this degree might have not been important enough to compromise cell growth and HN detection at least after 24 h.

Another aspect was the low level of HN protein detection. An explanation would be that the HN protein had been degraded by *A. oryzae* extracellular proteases, and certainly, it had. However, this difference between amylase and HN expression might also be influenced by the site of expression cassette integration. If the expression cassette integrated at the *argB* locus or elsewhere instead of the *amy3* locus, the amount of amylase detected would not only be the result of transcription of the *amy3*-HN integrated cassette sequence, but also transcription of the resident *A. oryzae amy3* gene. Studies of transformant mRNA levels in cells grown in induced and non-induced conditions might also be useful in demonstrating regulation of HN synthesis and the extent of mRNA stability.

4.4 Time course of HN production by transformant T1560gpdglaASSHN-3

10^8 spores of transformant T1560gpdglaASSHN-3 were inoculated into 10 vials of 50 ml MM containing 1 %

glucose as the sole carbon source. These cultures were incubated at 34°C, with agitation, for 12-14 h. Mycelia from 5 vials were washed with 1 % maltose MM and reinoculated into fresh MM containing 1 % maltose as the sole carbon source. The five other vials were washed with 1 % glucose MM and reinoculated into fresh MM containing 1 % glucose as the sole carbon source. All cultures were reincubated as before. One culture from each condition were removed after 1.5, 3, 6, 9 and 24 h of incubation for analysis. Three 1 ml aliquots of these culture filtrates were lyophilized. The resulting powder was resuspended and loaded in SDS-PAGE in triplet. One gel was stained with Coomassie blue and destained with methanol-acetic acid solution to enable protein visualization. Proteins from two gels were transferred onto nitrocellulose filters. One filter was probed with anti-TAG MAb and protein A conjugated to peroxidase and the other was probed with anti-*A.niger* GLAA pAb and anti-rabbit IgG conjugated to alkaline phosphatase. Protease inhibitors (PMSF and EDTA to 1 mM final concentration) were added to the remainder of each culture filtrate (about 48 ml) and this was submitted to immunoprecipitation using anti-TAG MAb (See Materials and Methods Section 2.8.4). Immunocomplexes were western blotted and probed with anti-TAG MAb and protein A conjugated to peroxidase. HN detection was carried out using ECL reagents. The results are shown in Fig. 3.4.8.

Figure 3.4.8 Time course of HN production by transformant T1560gpdglaASSHN-3 . Transformant T1560gpdglaASSHN-3 were grown in MM containing 1 % glucose as the sole carbon source, at 34°C, overnight. Mycelia were harvested and reinoculated into fresh MM containing either 1 % glucose or 1 % maltose as the sole carbon source. Cultures were harvested after 1.5, 3, 6, 9, and 24 h of incubation at 34°C, with agitation. Proteins contained in 1 ml of culture filtrates were separated in a 12.5 % SDS-polyacrylamide gel. The gels were either stained with Coomassie blue and destained with methanol-acetic acid solution (Panel A) or western blotted. The filter was probed with anti-*A.niger* GLAA pAb and anti-rabbit IgG conjugated to alkaline phosphatase. Glucoamylase was detected by NTB and BICP reagents (Panel C). Western blotted immunocomplexes obtained by immunoprecipitation using anti-TAG MAb were probed with anti-TAG MAb and protein A peroxidase conjugated (see text for details) (Panel B). The arrow on the right of Panel A shows the *A.niger* glucoamylase band position.

Lane M - Molecular weight markers, sizes are in kDa

Lane C - Denatured HN immunocomplex (SDS-PAGE) or tag-labelled protein marker (immunoblot).

Lane 1 - T1560gpdglaASSHN-3 grown in glucose, overnight

Lane 2 - T1560gpdglaASSHN-3 grown in glucose, for 1.5 h

Lane 3 - T1560gpdglaASSHN-3 grown in glucose, for 3 h

Lane 4 - T1560gpdglaASSHN-3 grown in glucose, for 6 h

Lane 5 - T1560gpdglaASSHN-3 grown in glucose, for 9 h

Lane 6 - T1560gpdglaASSHN-3 grown in glucose, for 24 h

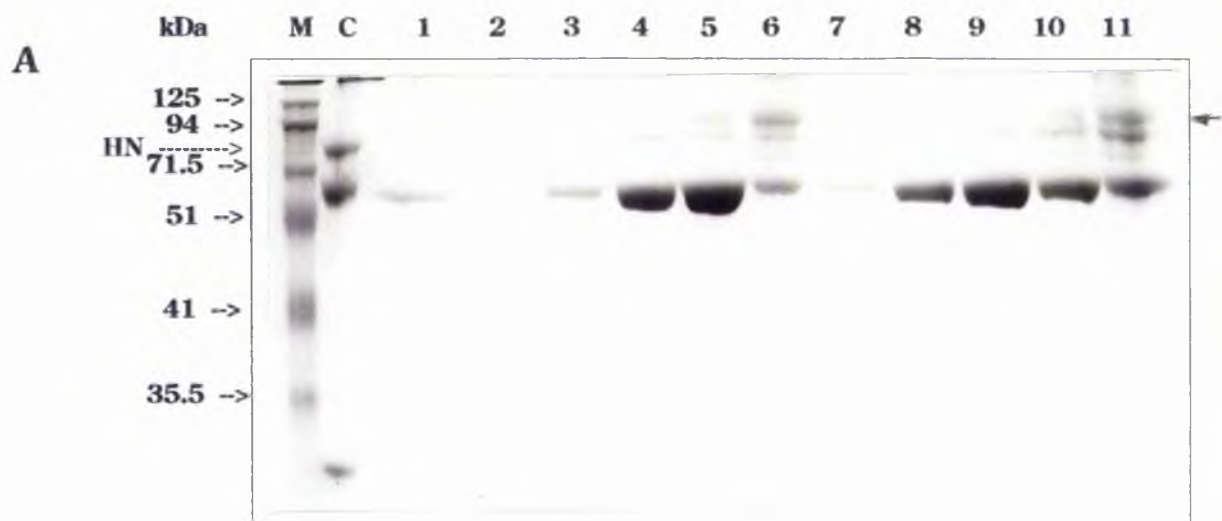
Lane 7 - T1560gpdglaASSHN-3 grown in maltose, for 1.5 h

Lane 8 - T1560gpdglaASSHN-3 grown in maltose, for 3 h

Lane 9 - T1560gpdglaASSHN-3 grown in maltose, for 6 h

Lane 10 - T1560gpdglaASSHN-3 grown in maltose, for 9 h

Lane 11 - T1560gpdglaASSHN-3 grown in maltose, for 24 h



Recombinant *A.niger* glucoamylase activity does not seem to be induced by maltose, as expected, since its synthesis is under the control of the *A.nidulans* *gpdA* promoter (Fig. 3.4.8, Panel A, SDS-PAGE). However, there were signs of its proteolytic degradation after 6 h of incubation in both glucose and maltose medium which increased after 24 h of incubation (Fig. 3.4.8, Panel C, western blot probed with anti-*A.niger* GLAA pAb).

HN protein was not detected in a western blot of proteins obtained from in 1 ml of this culture filtrates (Lucena, unpublished). The detection of HN protein involved the immunoprecipitation of approximately 50 ml of culture filtrate. In glucose medium, HN protein was detected at 1.5 h of incubation reaching the maximum at 3 h, but its detection decreased by approximately 50 % at 6 h and again at 9 h. Finally, no HN was detected at 24 h. However, although HN expression was under the control of a non-induced promoter, a different profile was obtained in maltose medium, where HN protein was detected only at 3 h of incubation (Fig. 3.4.8, Panel B, western blotted immunocomplexes probed with anti-TAG MAb).

The results showed indicate: (1) *A.niger* glucoamylase was expressed and secreted into the medium and its proteolytic degradation took place after 6 h of incubation. (2) HN was detected at maximum level after 3 h of incubation followed by a significant decrease. The decrease in HN detection coincided with the

detection of a smeared glucoamylase band on western blot, such a smeared band is characteristic of protein degradation. However, degradation could not be identified on an HN immunoblot, probably, because the removal of the TAG-sequence by proteases lead to non-detection of the smeared HN band. (3) Finally, the HN was found secreted in maltose medium only at 3 h of incubation. Comparing the HN detection profile in glucose medium, at this time the level of secreted HN achieved the maximum. It is speculated if maltose medium could have facilitated the production of extracellular proteases. Consequently, HN degradation would have been more pronounced.

4.5 Aspects of HN folding

The production of authentic viral proteins is essential for development of vaccines. In this section, the capacity of *A.oryzae* to produce the mature HN molecule correctly folded was assessed.

10^7 spores from five day-old cultures of transformants T1560amyHN-8, T1560amyASSHN-18, T1560gpdglaHN-6 and T1560gpdglaASSHN-3 inoculated into 50 ml of MM containing 2 % maltose as the sole carbon source and 10 mM ammonium as the sole nitrogen source. All cultures were incubated at 34°C, with agitation, for 13 h. Mycelia were removed by filtration and the culture filtrates were used for HN immunoprecipitation.

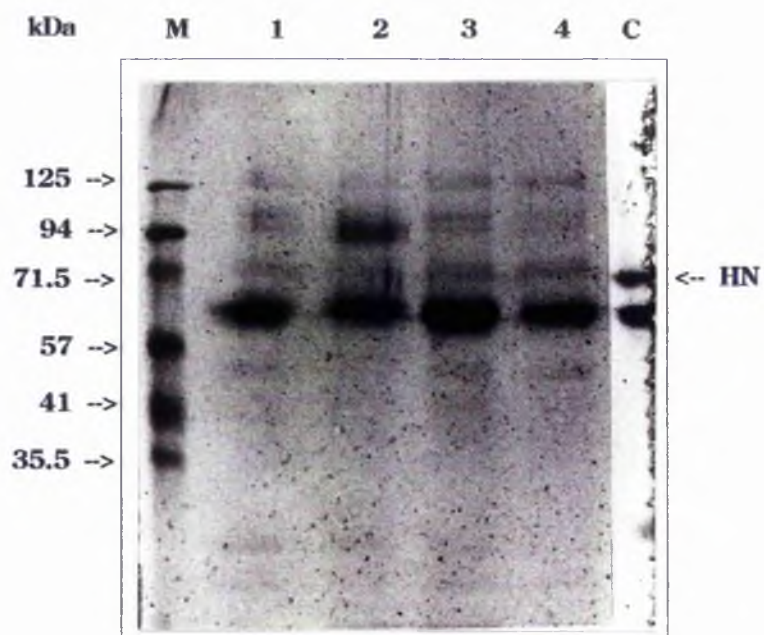
Immunocomplex was prepared with a conformational anti-HN MAb and *Staphylococcus* protein A solution (for details see Materials and Methods Section 2.8.4). These immunocomplexes were added to each 50 ml culture filtrates. Immunoprecipitation was carried out at 4°C for 4 h. Immunocomplexes were separated by 10 % SDS-PAGE and transferred onto a nitrocellulose membrane. This immunoblot was probed with anti-TAG MAb and protein A conjugated to peroxidase.

In Figure 3.4.9 Panel A, the immunoblot result shows that the HN protein expressed in *A.oryzae* was recognized and immunoprecipitated by a conformational anti-HN MAb. A band of approximately 65 kDa in size was detected in transformants T1560amyASSHN-18 and T1560gpdglaASSHN-3. Another band of a size ranging from 94 kDa to 125 kDa was also detected in T1560gpdglaASSHN-3 which probably represented the fused glucoamylase-HN protein. No bands were identified in T1560amyHN-8 and T1560gpdglaHN-6. However, there is a possibility that the HN protein had been expressed in T1560amyHN-8 and T1560gpdglaHN-6 as a membrane protein. In support of this hypothesis is the fact that the expression cassettes used to give transformants T1560amyHN-8 and T1560gpdglaHN-6 contain the hydrophobic N-terminal region of the HN protein.

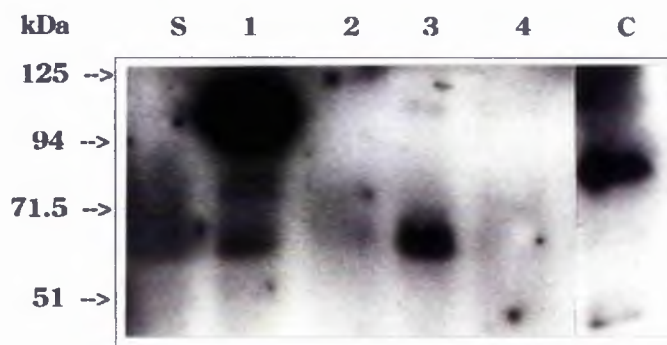
Figure 3.4.9 Analysis of HN molecule conformation. HN sequence containing- *A.oryzae* transformants were grown in MM containing 2 % maltose, at 34°C, overnight. Proteins contained in 1 ml of culture filtrates were separated in a 10 % SDS-polyacrylamide gel (Panel A). Approximately 50 ml of culture filtrate was submitted to an immunoprecipitation with anti-HN 173 MAb, a conformational antibody, followed by SDS-PAGE and immunoblotting. The immunoblot was probed with anti-TAG MAb and protein A conjugated to peroxidase. HN was detected by chemiluminescence method (Panel B).

Lane M - Molecular weight markers, sizes are in kDa
Lane C - Denature HN immunocomplexes (PAGE)
tag-labelled protein marker (Western blot)
Lane S - anti-HN MAb plus *S.aureus* Protein A
Lane 1 - T1560gpdglaASSHN-3
Lane 2 - T1560gpdglaHN-6
Lane 3 - T1560amyASSHN-18
Lane 4 - T1560amyHN-8

A



B



4.6 Genetic analysis of transformants

In order to examine if the low expression level of HN protein was also influenced by the number of copies of expression cassette integrated, the number of HN cDNA copies integrated in 5 μ g genomic DNA was estimated.

4.6.1 Copy gene equivalent

pAN56-ASSHN was measured by diaminobenzoic (DABA) fluorescence assay (Gurney & Gurney, 1984) and diluted in order to provide samples containing 1, 5, 10, 50 and 100 HN cDNA copies when compared to 5 μ g of genomic DNA. Acetamide prototrophic transformants T537amyHN-10 and T537amyASSHN-18, and arginine prototrophic transformants T1560amyHN-8, T1560amyASSHN-18, T1560gpdglaHN-6 and T1560gpdglaASSHN-3 were used in this experiment (for identification of transformants see Table 3.4.3). Genomic DNA from each of these transformants was dot blotted onto nylon membrane at concentration of 0.5, 0.75, 1 and 5 μ g. This filter was probed with the BamHI fragment internal to the HN cDNA sequence.

Surprisingly, T537amyHN-10 and T537amyASSHN-18 did not hybridize to the HN DNA specific probe suggesting that the integrated expression cassette has been deleted. T1560amyHN-8 seemed to contain a few (1 or 2) copies of integrated HN cDNA sequence. In contrast, T1560amyASSHN-18 incorporated approximately 100 copies

of HN cDNA sequence, while T1560gpdglaHN-6 and T1560gpdglaASSHN-3 seemed to have integrated more than 100 copies (Figure 3.4.10).

4.6.2 Integrity of the integrated expression cassette

In order to investigate possibility of deletions of the integrated HN cDNA sequence, genomic DNA from those transformants analysed in Section 4.6.1 were submitted to PCR for HN fragment amplification. The HN encoding DNA fragment was amplified using (1) a specific 5' primer HNback and 3' primer GEXFOR and (2) 5' and 3' primers homologous to the *amy3* gene sequences located in the region 50 base pairs upstream and 350 base pairs downstream of the stop codon, respectively (the primer designs are shown previously in Figs. 3.2.2 and 3.3.1).

The HN cDNA-PCR fragment amplified from T1560amyASSHN-18 genomic DNA was similar in size to that amplified from pGEXCHNTAG. Moreover, the results obtained from PCR using primers homologous to the *amy3* gene suggested that integration did not occur at the *amy3* locus, judged by amplification of the 0.4 kb fragment which corresponds to the natural 3'end of *A.oryzae amy3* gene (Figure 3.4.11).

The HN cDNA fragment amplified from T1560gpdglaASSHN-3 genomic DNA was larger (2.4 kb) than the HN fragment amplified from pGEXCHNTAG (1.6 kb)

Figure 3.4.10 Copy gene equivalent determination. The concentration of pAN56-ASSHN was measured by DABA fluorescence assay (Gurney & Gurney, 1984). Further dilution of pAN56-ASSHN was carried out in order to obtain an equivalent of 1, 5, 10, 50 and 100 HN cDNA copies integrated in 5 μ g of genomic DNA. Genomic DNA from transformants were dot blotted onto nylon membrane at concentration of 0.5, 0.75, 1 and 5 μ g (from the left to the right on the picture). The filters were probed with a *Bam*HI fragment of HN cDNA sequence. Hybridization was carried out under stringent conditions.

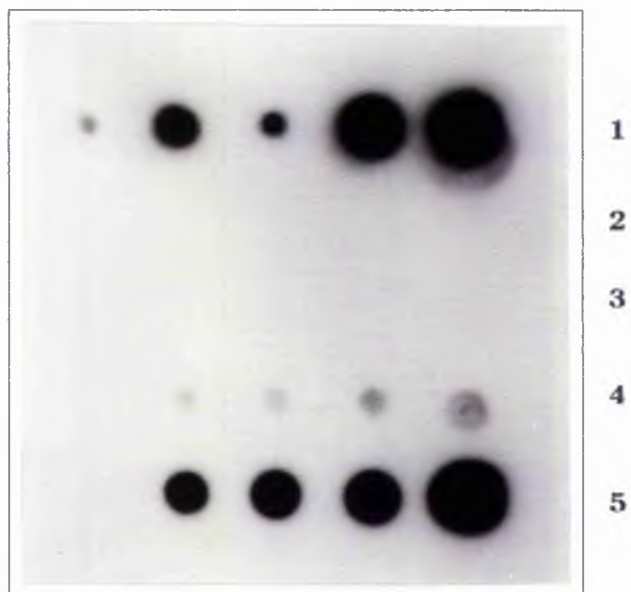
Panel A - Analysis of few transformants which contain amylase-HN DNA sequences on expression cassette.

Line 1 - 1, 10, 5, 50 and 100 copies of HN gene
Line 2 - Transformant T537amyHN-8 genomic DNA
Line 3 - Transformant T537amyASSHN-18 genomic DNA
Line 4 - Transformant T1560amy3HN-10 genomic DNA
Line 5 - Transformant T1560amyASSHN-18 genomic DNA
Line 5, column 1 - *A.oryzae* strain 1560-6, genomic DNA
(5 μ g), a negative control.

Panel B - Analysis of few transformants which contain glucoamylase-HN DNA sequences on the expression cassette.

Line 1 - 1, 5, 10, 50 and 100 copies of HN gene
Line 2 - Transformant T1560gpdglaHN-6 genomic DNA
Line 3 - Transformant T1560gpdglaASSHN-3 genomic DNA
Line 3, column 1 - *A.oryzae* strain 1560-6, genomic DNA
(5 μ g), a negative control.

A



B

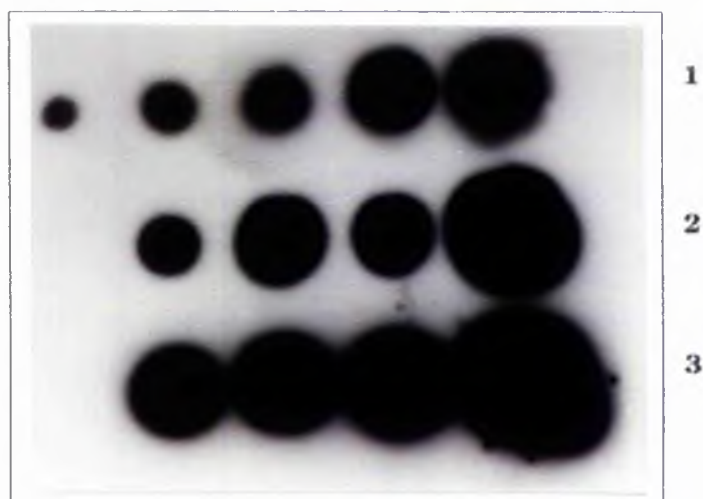
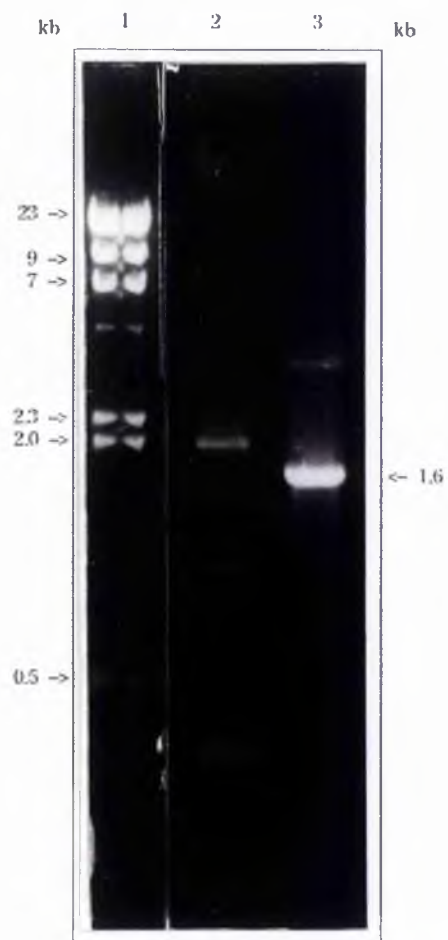


Figure 3.4.11 Polymerase chain reaction (PCR) analysis of the HN cDNA sequence integrated into chromosome of *A.oryzae* transformants . Analysis of ASSHN fragment integrated into genomic DNA of T1560amyASSHN-18 transformant.

- Lane 1 - Markers, *Hind*III digested lambda DNA
- Lane 2 - Fragment generated by amylase primers
- Lane 3 - Fragment generated by ASSHNback and GEXFOR primers



(Lucena, unpublished). However, HN cDNA-PCR fragment amplified from the pAN56-ASSHN expression vector, which was used in transformation experiments, was also 1.6 kb long (Figure 3.3.9). This result suggested that insertion of a small fragment of *A.oryzae* genomic DNA could have occurred, probably by recombination.

In contrast, no HN cDNA PCR fragment was amplified using genomic DNA from T1560amyHN-8, T1560gpdglaHN-6, T537amyHN-10 or T537amyASSHN-18 as the template (Lucena, unpublished). It is unlikely T537amyHN-10 and T537amyASSHN-18 are false-negative HN-transformants (see Figure 3.4.2). However, the genomic DNA from these transformants did not hybridize to the HN specific probe for a second time (Figure 3.4.10) nor were able to function as template for HN fragment amplification. This might have been a result from a complete or partial deletion of the expression cassette integrated into the host chromosome. On the other hand, there is a evidence that the recombinant *A.niger* glucoamylase has been expressed in transformant T1560gpdglaHN-6 (Figure 3.4.3). A small deletion at the 3'end of the integrated expression cassettes could result in a lack of template homology to the 3'primer. Thus, the integrated expression cassette sequences failed to amplify HN sequences but are functional for glucoamylase synthesis.

4.7 Influence of pH on HN production

In order to examine the influence of the pH of the liquid medium on HN production (result of HN synthesis and HN degradation), another time course of HN production was carried out using the T1560amyASSHN-18 strain.

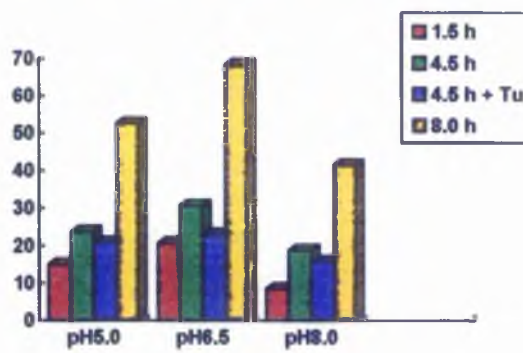
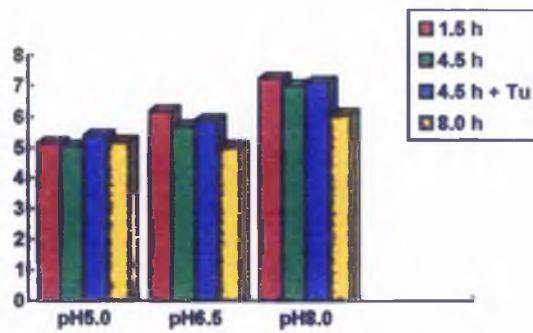
Approximately 10^7 spores from a five day-old culture were inoculated into 25 ml of MM (2 % maltose, 10 mM ammonium) buffered with (1) 10 mM sodium citrate buffer pH 5.0, (2) 10 mM sodium phosphate buffer pH 6.0, or (3) 10 mM Tris-HCl buffer pH 8.0, each in four vials. All cultures were incubated at 34°C, with agitation, for 14-16 h. Mycelia were washed and reinoculated into 25 ml of the same fresh MM. In order to examine if the recombinant *A.oryzae* HN protein is glycosylated to one vial of each set of MM buffered at pH 5.0, pH 6.5 or pH 8.0 was added tunicamycin at the final concentration of 4 µg / ml in order to block glycosylation in the Golgi compartment. All these cultures were incubated as before. One vial of each set of cultures buffered at different pH was harvested after 1.5 h, 4.5 h and 8 h of incubation. The cultures supplemented with tunicamycin were harvested after 4.5 h of incubation when, on basis on the time course experiment (Fig. 3.4.7), HN secretion is high. Two aliquots of 1 ml of each culture filtrate were lyophilized overnight. These samples were used in SDS-PAGE studies. The remainder of the culture filtrate (about 23 ml) was submitted to immunoprecipitation with

anti-TAG MAb. Western blotted immunocomplexes were probed with anti-TAG MAb and protein A conjugated to peroxidase. HN protein was detected by chemoluminescence (ECL reagents).

Although the incubation media were buffered to pH 5.0, pH 6.5 or pH 8.0, the final medium pH varied along the incubation time. The acidification of the media was more accentuated in pH 6.5 and pH 8.0 buffered cultures. Furthermore, mycelial growth was reduced at pH 8.0 buffered medium (Figure 3.4.12).

Surprisingly, the profile of HN secretion changed by growing the transformant in buffered medium. HN protein was only detected after 1.5 h incubation in buffered medium pH 5.0 and pH 6.5 but only after 8 h incubation in buffered medium pH 8.0 when the pH reduced to 6.0 (Figure 3.4.13, Panel B). The level of HN secreted was insignificant that was only possible to be detected by immunoprecipitation. The influence of tunicamycin on HN secretion could not be analysed, as HN production was affected by the controlled extracellular pH and no HN secretion was observed either in the presence or absence of tunicamycin at 4.5 h. Possibly, in these conditions, the proteolytic degradation of HN protein was greater. In mammalian cells, a secreted form of HN protein is retained inside the cells by addition of tunicamycin in the medium.

Figure 3.4.12 The influence of the extracellular pH on HN production. Mycelium from transformant T1560amyASSHN18 was grown in pH 5, pH 6.5 or pH 8 buffered MM containing 2 % maltose as the sole carbon source, at 34°C for 1.5, 4.5 and 8 h. Tunicamycin (0.4 µg/ml) was added to one culture of each different set of buffered MM. The pH of the culture filtrates and the biomass produced are shown in the table. The differences of the final pH's and biomass in these cultures are shown in a graphic representation (top and bottom respectively).

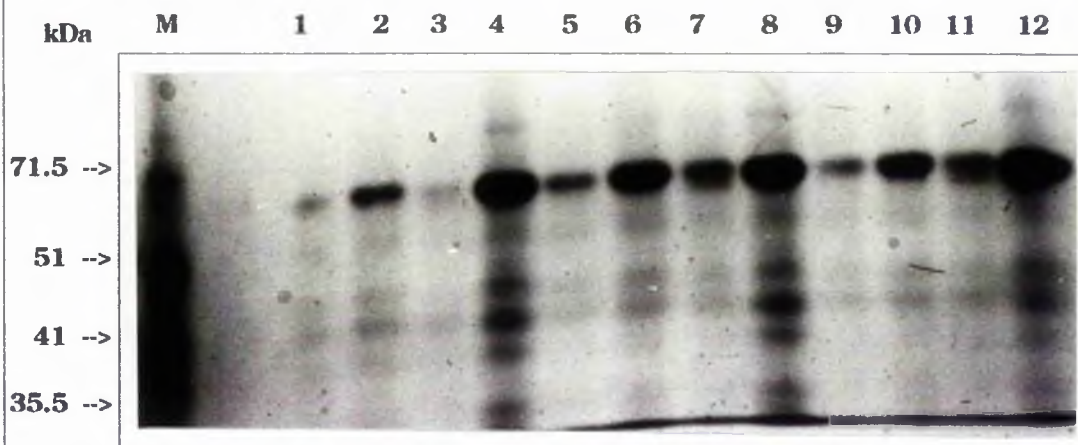


Initial pH	Incubation Time (h)	Final pH	dry weight (mg)
5.0	1.5	5.14	15.0
5.0	4.5	5.04	23.7
5.0	4.5 (+ Tu)	5.39	20.8
5.0	8.0	5.19	52.7
6.5	1.5	6.17	20.6
6.5	4.5	5.68	30.7
6.5	4.5 (+ Tu)	5.88	23.0
6.5	8.0	5.03	68.4
8.0	1.5	7.24	8.5
8.0	4.5	6.99	18.8
8.0	4.5 (+ Tu)	7.11	15.9
8.0	8.0	6.07	41.6

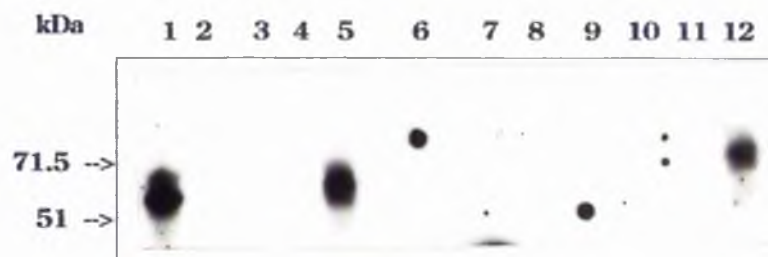
Figure 3.4.13 Studies of the influence of the extracellular pH on HN protein detection. Transformant T1560amyASSHN-18 was grown in MM containing 2 % maltose as the sole carbon source and buffered to pH 5, pH 6.5 or pH 8. Incubation was carried out at 34°C for 1.5, 4.5 and 8 h. Tunicamycin (0.4 µg/ml) was added to one culture of each different set of buffered MM. Proteins contained in 1 ml of culture filtrates were separated in 10 % SDS-polyacrylamide gels. The gels were either stained with Coomassie blue and destained with methanol-acetic acid solution (Panel A) or western blotted. The filter was probed with anti-TAG MAb and protein A conjugated to peroxidase. HN protein was detected by ECL reagents (Panel B).

Lane M - Molecular weight markers, sizes are in kDa
Lane 1 - T1560amyASSHN-18 grown in MM pH 5, for 1.5 h
Lane 2 - T1560amyASSHN-18 grown in MM pH 5, for 4.5 h
Lane 3 - As Lane 2, in presence of tunicamycin
Lane 4 - T1560amyASSHN-18 grown in MM pH 5, for 8 h
Lane 5 - T1560amyASSHN-18 grown in MM pH 6.5, for 1.5 h
Lane 6 - T1560amyASSHN-18 grown in MM pH 6.5, for 4.5 h
Lane 7 - As Lane 6, in presence of tunicamycin
Lane 8 - T1560amyASSHN-18 grown in MM pH 6.5, for 8 h
Lane 9 - T1560amyASSHN-18 grown in MM pH 8, for 1.5 h
Lane 10 - T1560amyASSHN-18 grown in MM pH 8, for 4.5 h
Lane 11 - As Lane 10, in presence of tunicamycin
Lane 12 - T1560amyASSHN-18 grown in MM pH 8, for 8 h

A



B



4.8 Improvement of HN production

In an attempt to obtain a higher level of secreted HN, transformant T1560amyASSHN-18 was grown on solid state culture. This strategy was adopted based on the fact that *Aspergillus oryzae* has been grown on rice to produce amylase for many years (Shah et al., 1991) and this transformant contains the amylase-HN construction.

10^8 spores from five day-old cultures of transformant T1560amyASSHN-18 were used to inoculate into four vials of MM containing 1 % glucose and incubated at 34°C, with agitation, for 14 h. Mycelium from each culture was harvested, washed with saline-TWEEN 80 solution and dried by pressing against sterile filter paper. These mycelia were re-inoculated on to solid state media (10 g of bran, rice or corn) and also into 50 ml MM containing 2 % maltose as control. These cultures were incubated at 30°C for 24 h. Each solid culture was washed with 50 ml of sterile water by shaking at 4°C for 4 h. A clear washing solution was obtained by filtration through sterile muslin and centrifugation at 8000 x g for 5 min. Two 1 ml aliquots from these washes and from maltose culture filtrate were lyophilized. A SDS-PAGE was run in duplicate, one gel was stained with Coomassie blue and destained with methanol-acetic acid solution and the other gel was submitted to western blotting. Blotted proteins were probed with anti-TAG MAb and protein A conjugated to peroxidase. The HN protein was detected by

chemoluminescence (ECL reagents). The result is shown in Figure 3.4.14.

Two prominent protein bands were detected in maltose medium, which represent the *A.oryzae* amylase (55 kDa) and glucoamylase (71 kDa) glycoproteins. From the growth on bran mainly amylase was detected, while on rice another small protein band of approximately 20 kDa was revealed. In contrast, several protein bands could be visualized in the sample grown on corn. The larger band could contain, *a priori*, the mature form of HN. However, western blot analysis revealed only a weak band in the sample from rice medium. Therefore, those bands from corn medium represented fungal proteins. The level of secreted HN should be considered less than 1 μ g per litre since it was not possible to detect the HN protein band in Coomassie stained gel. Nevertheless, the relevant aspect of this experiment was the HN detection in western blot without immunoprecipitation which leads us to believe that higher levels could be achieved by manipulating solid state growth conditions.

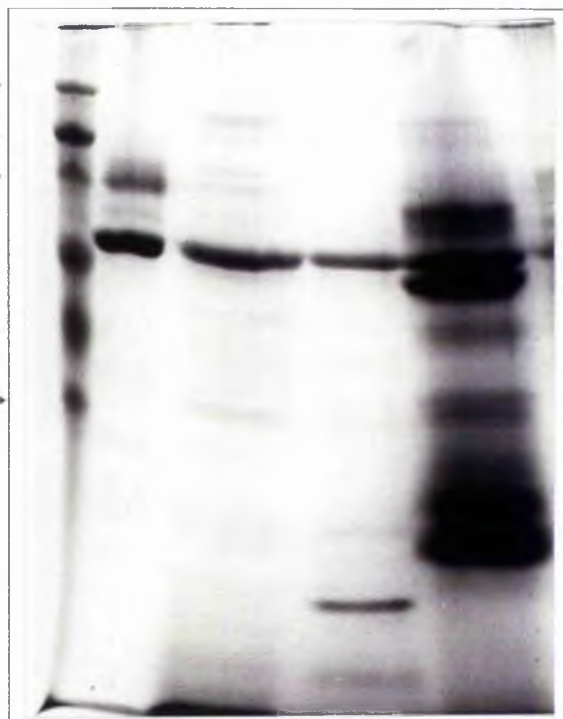
Figure 3.4.14 Studies on solid state culture. Transformant T1560amyASSHN-18 was grown on wheat bran, rice or corn and also in liquid MM containing 2 % maltose as the sole carbon source. Solid cultures were washed with sterile distilled water. Proteins contained in 1 ml of these washes and the culture filtrate were separated in 12.5 % SDS-polyacrylamide gel. The gels were either stained with Coomassie blue and destained with methanol-acetic acid solution (Panel A) or western blotted. The filter was probed with anti-TAG MAb and protein A conjugated to peroxidase. HN protein was detected by chemiluminescence method.

- Lane M - Molecular weight markers, sizes are in kDa
- Lane C - tag-labelled protein marker (western blot).
- Lane 1 - T1560amyASSHN-18 grown in liquid medium
- Lane 2 - T1560amyASSHN-18 grown on bran solid medium
- Lane 3 - T1560amyASSHN-18 grown on rice solid medium
- Lane 4 - T150amyASSHN-18 grown on corn solid medium
- Lane 5 - Denatured HN immunocomplex. HN produced by T1560amyASSHN-18 and immunoprecipitated by a conformational anti-HN MAb.

A

kDa M 1 2 3 4

125 ->
94 ->
71.5 ->
51 ->
41 ->
35.5 ->



B

kDa C 1 2 3 4 5

125 ->
94 ->
71.5 ->
57 ->
41 ->
35.5 ->



CHAPTER 4

CONCLUSION

Expression and secretion of the human gene interleukin-6 in the filamentous fungus *Aspergillus oryzae* was achieved using a plasmid vector construction containing the *A.niger glaA* (glucoamylase [GLAA]) coding region linked to the hIL-6 cDNA sequence by a KEX2-motif encoding DNA fragment. hIL-6 containing expression vectors were introduced by means of co-transformation experiments using the *A.nidulans argB* and *amdS* genes or the *A.oryzae niaD* gene, as the gene transfer and selection system. First, nitrate non-utilising (*niaD*⁻) mutants of *A.oryzae* strains IMI 144242 and RIB40 and *A.sojae* strain RIB1045 transformed with an hIL-6 containing plasmid were found to secrete the heterologous GLAA, but recombinant hIL-6 was not observed in culture filtrates. Nevertheless, the nitrate utilising hIL-6 transformant TD14-325, harbouring expression cassettes without KEX2-site, was found to secrete a hybrid GLAA/hIL6 protein. Second, arginine utilising hIL-6 transformants of *A.oryzae* strain 1560-6 designated T1560-gla15 and T1560-gpd1 were found to secrete a hybrid GLAA/hIL6 protein as well as a free form hIL6 in the culture filtrates. Third, acetamide utilising hIL-6 transformants of *A.oryzae* strain 1560-6 do not show hIL-6 in the culture filtrates.

One major problem of expressing foreign proteins in filamentous fungi appears to be proteolytic degradation of the produced foreign protein as judged by studies using *A.nidulans* and *A.niger* (Contreras et al., 1991; van den Hondel et al., 1992; Roberts et al., 1992; Archer et al., 1992). Indeed, *A.oryzae* has been shown to produce a copious amounts of extracellular proteases (Tatsumi et al., 1989 and 1991; Cheevadhanarak et al., 1991 and Ramamurthy & Kothari, 1993). The *A.oryzae* produced hIL-6 protein was shown to be rapidly degraded in culture medium. In contrast, the heterologous GLAA was found to be present in the culture medium and at high levels. This suggests that *A.oryzae* proteases recognized and degraded the foreign hIL-6 protein and that the *A.niger* GLAA might have been degraded slower. This may be due to its homology (67 %) with host glucoamylase (Hata et al., 1991). Furthermore, hIL-6 present in the GLAA/hIL6 hybrid protein seems to be partially or wholly protected from proteolytic degradation.

The production of hIL-6 by *A.oryzae* transformant strain T1560-gla15 was improved by generating 2-deoxy-D-glucose resistant mutants of this strain. Although higher levels of secreted hIL-6 were obtained, hIL-6 appered to be degraded rapidly in the medium. The maximum level of secreted hIL-6 was found to be

approximately 4 mg/l as judged the levels observed in SDS-polyacrylamide gels and western blots.

A plasmid expression vector harbouring the entire *A.oryzae amy3* (α -amylase) gene was constructed. Mutation in the *amy3* gene stop codon was carried out, by PCR technology, in order to create cloning sites and a KEX2-site. *A.oryzae* contains a KEX2-like endopeptidase which recognizes and cleaves on the carboxyl side of dibasic amino acid peptides, such as Arg-Arg combination. Thus, nucleotides encoding a KEX2-motif was included in the expression cassette DNA sequences - upstream of the foreign cDNA sequences, in an attempt to obtain a secreted foreign protein which would be correctly processed at its N-terminus.

In addition high yields, the production of authentic foreign protein, in terms of glycosylation pattern and molecule conformation, is obviously very desirable. Pharmaceutically important proteins such as human hormones and viral envelope proteins are normally glycosylated. Altered glycosylation in recombinant human hormones might lead to abnormal biological activity or adverse immunological responses. Differences in carbohydrate structures of a recombinant viral glycoprotein might induce a different degree of antigenicity. Therefore, vaccines produced with non-authentic viral glycoproteins could confer little or no

protection against the respective viral infection. *A.oryzae* has been shown to be capable to secrete foreign protein, such as human lactoferrin in similar fashion to native lactoferrin in terms of glycosylation and glycosylation profiles (Ward et al., 1992). In the experiments presented here the feasibility of *A.oryzae* to secrete authentic viral glycoprotein was assessed using the Simian virus 5 Haemagglutinin-neuraminidase (HN) glycoprotein as a model. For such studies, the entire HN cDNA sequence was generated by PCR technology (designated HN-DNA fragment). However, as HN is a membrane bound type II glycoprotein, it might not be secreted due to its anchor-domain. Thus, a deletion of the first 108 bp of its cDNA sequence, corresponding to the hydrophobic N-terminal region, was carried out in a second PCR reaction (designated ASSHN-DNA fragment). Both HN and ASSHN DNA fragments were inserted in-frame downstream of the *A.oryzae amy3* gene by a KEX2-motif encoding DNA sequence in plasmid vector designated pSTA1210, generating pSTA1210-HN and pSTA1210-ASSHN respectively. Both fragments were also inserted in-frame downstream of the *A.niger glaA* coding region via KEX2-encoding DNA in plasmid vector pAN56-30, generating pAN56-HN and pAN56-ASSHN respectively. The expression of *glaA* gene is under the control of the constitutive *A.nidulans gpdA* promoter in pAN56-30. Both HN- and ASSHN- containing expression cassettes were introduced

into the *A.oryzae* *argB*⁻ mutant strain 1560-6 and *A.oryzae* *amdS*⁻ mutant strain Δ 537. Although *A.oryzae* transformants containing multiple copies of HN or ASSHN sequences were isolated, no significant production of the HN protein was observed. There were no obvious differences in expression levels using either the homologous *amy3* promoter or the *A.nidulans* *gpdA* promoter.

Unfortunately, we do not have vectors constructions with the HN and ASSHN DNA sequences fused either directly to the *amy3* or *glaA* promoter or by their signal sequences. Different levels of heterologous gene expression have been suggested to be related to different fusions between fungal and foreign sequences (Sleep et al., 1990). Although *Aspergillus* species recognize and process non-fungal signal sequences (Cullen et al., 1987 and Tsuchiya et al., 1992) the highest levels of expression have been achieved where the foreign cDNA sequences have been fused to the promoter by fungal signal sequences (Cullen et al., 1987; Carrez et al., 1990). The construction of a hybrid protein where a foreign protein is fused to a well secreted fungal protein has been shown to improve secretion levels. For instance, the secretion levels of calf chymosin and porcine pancreatic prophospholipase A2 in *A.niger* (Ward, 1989 and Roberts et al., 1992) and

human interleukin-6 in *A.nidulans* (Contreras et al., 1991) have been improved by fusing the N-terminus of these foreign proteins to the C-terminus of the *A.niger* glucoamylase.

Many factors other than proteolytic degradation of foreign protein affect heterologous gene expression. Transformants carrying multiple copies of the expression cassettes have been shown to produce high levels of heterologous proteins in many cases. However, production may not be proportional to the number of gene copies (Verdoes et al., 1993 and Ward, 1989). The site of integration has been suggested to be the reason in fact for low level of expression in transformants containing a high copy number (Miller et al., 1987). Moreover, the possible titration out of regulatory factors by the high number of integrated expression units might occur (Ward, 1989 and Kelly & Hynes, 1987).

Certain HN-transformants failed to yield amplified integrated expression cassette sequences when subjected to PCR technology. A plausible explanation is that the expression cassette sequences were damaged by rearrangements and/or deletion of their sequences. In this respect instability is often created by integration of such cassettes at high copy number. It was noticed that, in particular, the integrated expression cassettes of transformants of the *A.oryzae* Δ 537 recipient strain

were subjected to extensive damage. Such acetamide utilising HN-transformants, which were screened by Southern hybridization using an HN specific DNA probe, failed to do so in a second round of hybridization using the same probe.

Other potential factors involved in low expression of foreign genes are mRNA stability, translational efficiency and processing (Peberdy, 1993). Comparative analysis of codon usage of Simian virus 5 HN gene, human IL-6 gene, *A.niger glaA* gene, *A.oryzae amy3* and *glaA* genes (Table 4.1) shows that almost all codons are well represented in fungal genes and HN gene. However, fungal genes seem to have a markedly preference for G and C and also a selection against A at the third position in the codons. In contrast, in the HN encoding gene, there do not seem to be selection against any particular nucleotide at the third position, but there appears to be a preference for either U or A at this position. On the other hand, hIL6 gene does not use all the codons and, for example, UAU (Try), UCG (Ser) and GCG (Ala) are well represented in the fungal genes. However, the hIL-6 gene appears to have codon preference similar to that found in fungal genes for that codons represented. Rare codons are thought to be translated slower because their corresponding tRNA species are in short supply. Additionally, a cluster of repeated rare

Table 4.1 Codon Usage in genes reported in this study (see text for references).

Amino acid	Codon	<i>A.oryzae</i>		<i>A.niger</i>	SV5	Human
		amylase	glucoamylase	glucoamylase	HN protein	Interleukin 6
Phe	UUU	3	5	4	18	3
	UUC	12	18	18	6	8
Leu	UUA	1	2	0	11	0
	UUG	10	11	6	4	3
	CUU	4	6	3	11	3
	CUC	13	8	17	8	5
	CUA	3	3	2	11	2
	CUG	6	8	20	7	15
Ile	AUU	7	7	12	16	3
	AUC	20	17	11	18	5
	AUA	2	2	1	8	1
Met	AUG	11	4	3	15	6
Val	GUU	5	15	6	9	1
	GUC	12	15	15	6	1
	GUA	4	5	2	7	3
	GUG	10	12	19	8	3
Try	UAU	11	14	6	19	0
	UAC	24	13	21	9	3
ter	UAA	0	0	0	1	0
	UAG	0	1	1	0	1
His	CAU	3	3	0	1	1
	CAC	4	1	4	3	1
Gln	CAA	5	11	4	16	4
	CAG	15	18	13	18	10
Asn	AAU	3	10	6	28	3
	AAC	23	13	19	8	8
Lys	AAA	7	8	0	2	7
	AAG	13	8	13	8	7
Asp	GAU	20	15	21	11	4
	GAC	22	17	23	6	4
Glu	GAA	5	9	9	8	5
	GAG	7	17	17	6	11
Ser	UCU	4	9	16	18	2
	UCC	9	16	19	4	5
	UCA	5	7	4	13	2
	UCG	10	15	14	4	0
Pro	CCU	6	12	4	9	3
	CCC	7	7	10	6	1
	CCA	3	7	0	12	7
	CCG	5	4	8	2	0
Thr	ACU	9	11	20	19	3
	ACC	14	23	39	12	4
	ACA	10	7	5	23	5
	ACG	8	14	10	3	1
Ala	GCU	5	18	25	10	4
	GCC	15	18	19	8	9
	GCA	12	16	10	15	5
	GCG	10	16	11	1	0
Cys	UGU	7	5	3	8	2
	UGC	2	4	7	9	2
ter	UGA	1	0	0	0	0
Trp	UGG	12	17	19	9	1
Arg	CGU	1	3	4	3	0
	CGC	2	5	7	2	1
	CGA	2	6	4	6	1
	CGG	2	6	3	0	2
Ser	AGU	4	10	12	9	4
	AGC	6	19	23	10	6
Arg	AGA	0	2	1	5	4
	AGG	3	1	1	3	1
Gly	GGU	10	11	14	10	2
	GGC	18	15	22	9	1
	GGA	10	9	7	13	3
	GGG	5	12	4	8	1

codons has been shown to produce a frameshift frequency of 50 % (Anderson & Kurland, 1990). With heterologous expression cassettes integrated in high copy number, the sequestering of tRNA species at the rare codons might amplify this translational delay (Andersson & Kurland, 1990). Thus, HN codons which are rare in *A.oryzae* genes could have been translated slower. Moreover, the accumulated corresponding mRNA can be subjected to premature termination and further degradation. Furthermore, the protein synthesising pauses during the co-translational translocation process might lead to the degradation of the protein sections.

Recombinant *A.oryzae* HN protein was shown to be recognized by a conformational anti-HN MAb. A free form of HN protein was detected in filtrates from strains T1560gpdglaASSHN3 and T1560amyASSHN18. Although glycosylation of the *A.oryzae* recombinant HN protein was not determined in this case, it is speculated that the secreted form of HN was to some extent glycosylated. This prediction is based on the fact that glycosylation is essential for both membrane-bound and soluble forms of the SV5 HN protein folding and assembly at least in mammalian cells (Ng et al., 1990 and Parks & Lamb, 1990a). On the other hand misfolded HN molecules are normally retained inside the ER and further degraded (Parks & Lamb, 1990a). *A.oryzae* strain, transformed with

expression cassettes containing the entire HN cDNA sequence, did not appear to secrete the HN protein. The integrated expression cassettes of these transformants failed to yield amplified HN DNA sequences after PCR treatment. Nevertheless, in one particular transformant, namely T1560gpdglaHN-6, the expression cassettes appeared to be transcribed and translated since heterologous GLAA protein was found in the medium. There is a possibility that in this transformant HN protein was expressed as a membrane bound protein. Intracellular expression of HN protein was not assessed in this study.

Another aspect which should be considered in HN secretion is related to HN protein release from the hybrid protein. It has been shown that the hybrid protein processing by a KEX2-like endopeptidase is not essential for secretion *per se*. Both free and fused forms of foreign proteins have been found secreted. Moreover, there is a possibility that highly expressed hybrid proteins are secreted as fused proteins due to a saturation of the KEX2-like endopeptidase. However, it has been reported that restriction of intracellular transport of HN produced in mammalian cells, occurs when the ectodomain is malformed (Ng *et al.*, 1989, 1990; Parks & Lamb, 1990b). This may suggest that the molecular conformation adopted by the hybrid protein in the ER in *Aspergillus oryzae* might also affect HN folding and

secretion, although the fused GLAA-HN protein was found to be recognized by a conformational anti-HN MAb.

By manipulating the growth conditions, the secretion levels of both hIL6 and HN protein were improved. The production of heterologous proteins by *A.oryzae* grown under fungal solid state culture were assessed. hIL6 transformants were found to secrete a fused form of hIL6 protein when grown on rice or wheat bran media. No free form of hIL6 protein was found which suggested that in these conditions the production of extracellular proteases might also be high (Ramamurthy & Kothari, 1993). It appeared that the duration and conditions of harvesting the recombinant protein produced by solid state culture were essential in avoiding proteolytic degradation of the heterologous protein. The maximum level of secreted HN detected was achieved by growing strain T1560amyASSHN18 on rice based solid state medium. HN was detected in a western blot of proteins in 1 ml aliquots from solid state culture filtrates, whereas previously HN detection was only possible by immunoprecipitation in 50 ml of liquid culture filtrate. Nevertheless, the HN protein can not be detected in Coomassie stained gels. The yield of HN protein extracted with 50 ml of sterile water from 10 g rice based solid state medium is estimated to be less than 1 μ g per ml.

Small scale of fed-batch fermentation was also assessed for hIL-6 production and preliminary results suggested that relatively high levels of secreted, free form hIL-6 can be obtained. Furthermore, it seems that the optimal fermentation mode, inoculum and environmental conditions including medium, pH, temperature, agitation and aeration will be determined by the type of product and the physiology of the organism involved (Borys et al., 1993).

Finally and in summary, hIL6 and the SV5 HN proteins have been expressed in *A.oryzae*, albeit at low levels. In addition to the proteolytic degradation of these heterologous proteins by *A.oryzae* extracellular and possibly intracellular proteases, other potential problems have been encountered, such as the inefficient translation and the inefficient folding or processing of the heterologous proteins in the secretory pathway. The knowledge of protein synthesis and secretion process in filamentous fungi will enable to understand the differences in secretion of homologous and heterologous proteins. Meanwhile the fact that *A. oryzae* is capable to secrete up to 20 g of native amylase per litre encourages us to continue to manipulate this extraordinary secretory machinery for the purpose of producing foreign proteins of human interest.

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